REMARKS

Claims 1-14, 20, 21, and 26-28 are pending in this application. Claim 21 has been canceled, and new claim 29 has been added. Claims 3-11 and 26 have been amended to add the phrase "or a pharmaceutically acceptable salt, solvate or hydrate thereof". Claim 7 has been amended to add the phrase "1, 2, 3, or 4". Claim 12 has been amended to delete one species. Claims 20 and 26 have been further amended to recite the disorders of claims 21 and 27. Claim 27 has been amended to recite dyslipidemia as the metabolic-related disorder. Support for the amendments can be found throughout the specification and original claims, for example, at page 34, lines 1-2 (pharmaceutically acceptable solvates and hydrates); at page 2, line 31 (pharmaceutically acceptable salt); at page 2, lines 17-18 (substituents of R_1 can be further optionally substituted by 1, 2, 3, or 4 substituents); and in original claim 26 (dyslipidemia and atherosclerosis as metabolic-related disorders). No new matter has been added. After entry of this amendment, claims 1-14, 20, and 26-29 will be pending in this application.

As a preliminary matter, Applicants thank the Examiner for withdrawing the restriction requirement.

I. Information Disclosure Statement

Applicants have filed a supplemental IDS herewith for the Examiner's consideration. Applicants thank the Examiner for consideration of the previously submitted IDS.

II. The Claims Are Enabled

A. Methods of Treatment

Claims 20-21 and 26-27 are rejected under 35 U.S.C. § 112, first paragraph, as allegedly failing to comply with the enablement requirement. As will be recognized, the enablement requirement of §112 is satisfied so long as a disclosure contains sufficient information that persons of skill in the art having the disclosure before them would be able to make and use the invention. *In re Wands*, 8 U.S.P.Q.2d 1400 (Fed. Cir. 1988) (the legal standard for enablement under §112 is whether one skilled in the art would be able to practice the invention without

undue experimentation). In this respect, the following statement from *In re Marzocchi*, 169 U.S.P.Q. 367, 369-370 (C.C.P.A. 1971), is noteworthy:

> As a matter of Patent Office practice, then, a specification disclosure which contains a teaching of the manner and process of making and using the invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented must be taken as in compliance with the enabling requirement of the first paragraph of § 112 unless there is reason to doubt the objective truth of the statements contained therein which must be relied on for enabling support. Assuming that sufficient reason for such doubt does exist, a rejection for failure to teach how to make and/or use will be proper on that basis; such a rejection can be overcome by suitable proofs indicating that the teaching contained in the specification is truly enabling.
>
> ... it is incumbent upon the Patent Office, whenever a rejection on this basis is made, to explain why it doubts the truth or accuracy of any statement in a supporting disclosure and to back up assertions of its own with acceptable evidence or reasoning which is inconsistent with the contested statement.

Thus, any assertion by the Patent Office that an enabling disclosure is not commensurate in scope with the protection sought must be supported by evidence or reasoning substantiating the doubts so expressed. *In re Dinh-Nguyen*, 181 U.S.P.Q. 46 (C.C.P.A. 1974); *In re Bowen*, 181 U.S.P.Q. 48 (C.C.P.A. 1974). Further, the proper standard for an enablement inquiry rests on whether one skilled in the art would be able to make and use the invention without undue experimentation. *In re Wands*, 8 U.S.P.Q.2d at 1404. Factors for consideration in determining whether undue experimentation is necessary to make and use the invention include 1) the quantity of experimentation necessary; 2) the amount of direction or guidance presented; 3) the presence or absence of working examples; 4) the nature of the invention; 5) the state of the prior art; 6) the relative skill of those in the art; 7) the predictability or unpredictability of the art; and 8) the breadth of the claims.

i. The nature of the invention and the breadth of the claims

The Office states that claims 20 and 26 encompass all metabolic-related disorders. Solely to advance prosecution, Applicants have amended claims 20 and 26 to recite the specific

disorders of claims 21 and 27, respectively. Claim 21 has been canceled, while claim 27 has been amended to recite dyslipidemia as the metabolic-related disorder. New claim 29 recites atherosclerosis as the metabolic-related disorders. Applicants respectfully assert that the scope of the amended claims is commensurate with guidance in the specification in light of the skill and knowledge of one of skill in the art.

ii. The state of the prior art

The Office cites Sparatore, et al., *Chem. & Biodiversity*, 3:385-395 (2006), stating that "certain benzotriazole compounds exhibit useful pharmaceutical properties as PPAR agonists and could be used to treat dyslipidemic type 2 diabetes or dyslipidemia without diabetes" (Office Action, page 4). The Office further cites Semple, et al. *J. Med. Chem.* 49:1227-1230 (2006), alleging that benzotriazole derivatives "can be used to treat dyslipidemia and atherosclerosis", but states that the "instant application is not directed to PPAR or GPR109b, but rather hRUP38" (Office Action, page 4). Applicants respectfully note that both the Sparatore and the Semple articles were published in 2006 which is after the filing date of the present application. Accordingly, these articles do not form part of the "state of the prior art". Applicants note, however, that hRUP38 is Applicants' internal reference number for the receptor more commonly known as GPR109b.

iii. The level of skill in the art

The Office has stated that the level of skill in the art is high. Without agreeing with the basis of this assessment, Applicants note that a high level of skill in the art will weigh in favor of a finding of enablement. *Wands*, 8 U.S.P.Q.2d 1406.

iv. The Predictability or unpredictability of the art

In a conclusory manner, the Office states that "the question [is] whether the compound of the present invention could be reliably and predictably extrapolated to patients with all metabolic-related disorders claimed" and concludes that "[t]here is no absolute predictability,

even in view of the high level of skill in the art" (Office Action, page 5). The Office further relies on general statements about the unpredictability of the pharmaceutical arts, without citing to any evidence for doubting the statements in the specification regarding the activity of the claimed compounds. Applicants respectfully note that the standard for enablement is not "absolute predictability", but whether the claimed invention can be made and used by a person of skill in the art without undue experimentation. Applicants further remind the Office that *Marzocchi* requires the Office to proffer more than just conclusory statements regarding why it doubts the assertions of the specification. Instead, the Office is required to "back up assertions of its own with acceptable evidence or reasoning which is inconsistent with the contested statement". *Marzocchi*, 169 U.S.P.Q. at 369-370. By relying on such conclusory statements regarding the generalized predictability of a large area like the pharmaceutical arts and convoluting the undue experimentation standard to one of "absolute predictability", the Office has failed to carry this burden.

v. The amount of direction or guidance presented and the presence or absence of working examples

While conceding that the specification "provides some data", the Office argues that the specification does "not show with any level of specificity how the instantly claimed compounds are agonists of hRUP38 or how they treat specific metabolic-related disorders" (Office Action, page 5). As to working examples, the Office states "there are no working examples of how a particular compound is used to treat a particular disorder or of the general mechanism of action used as a hRUP38 agonist" (Office Action, page 6). The Office notes a receptor binding assay in Example 8, but states that the assay does not provide specific data showing the binding capability of any of the instantly claimed compounds" (Office Action, page 6). The Office further points to the *in vivo* animal model in Example 7, but states that there is "very little data provided to show how the instantly claimed compounds are hRUP38 agonists or how the instantly claimed compounds act to treat a disorder" (Office Action, page 6). Solely to clarify the record,

Applicants respectfully note that Examples 7 and 8 are written in the present tense, indicating that these are prophetic examples.

Further, Applicants respectfully disagree that the specification does not provide working examples or that the specification does not provide guidance to one of skill in the art as to how to use the claimed methods. In particular, Applicants respectfully assert that the claimed methods are enabled, because:

(1) the working examples demonstrate that certain compounds of the invention decrease lipolysis in human adipocyte cells in a dose-dependent manner and reverse the cAMP elevating effect of forskolin;

(2) the art demonstrates a tie between a decrease in lipolysis and cAMP levels and treatment of metabolic-related disorders; and

(3) based on the working examples (1) and the teachings in the art (2), one of skill in the art would accept that the claimed compounds would be useful to treat metabolic-related disorders such as dyslipidemia and atherosclerosis.

First, the Office is respectfully directed to Example 3 and Figure 2 of the specification. Example 3 and Figure 2 show that both niacin and 1-isopropyl-1H-benzotriazole-5-carboxylic acid can inhibit isoproternol[1] stimulated lipolysis in adipocyte cells derived from human subcutaneous fat (see specification at page 46, lines 9-16 and Figure 2; see also, Semple, et al., "1-Alkyl-benzotriazole-5-carboxylic acids are highly selective agonists of the human orphan G-protein-coupled receptor GPR109b", *J. Med. Chem.*, 49(4):1227-1230 (2006) (cited in the supplemental IDS). Figure 2 shows that 1-isopropyl-1H-benzotriazole-5-carboxylic acid can inhibit isoproterenol stimulated lipolysis in a dose-dependent manner in a manner comparable to that of niacin. These data together demonstrate that certain compounds of the invention decrease lipolysis in human adipocyte cells in a dose-dependent manner. Applicants further direct the Office's attention to Figure 1, showing that 1-isopropyl-1H-benzotriazole-5-carboxylic acid can reverse the cAMP elevating effect of forskolin (see also, Semple at page 1228).

---

[1] Isoproternol acts to elevate cAMP levels by interaction with the β-adrenergic receptor, which, in turn, stimulates lipolysis. Lipolysis can be monitored by the resultant glycerol production, with decreasing glycerol production indicating inhibition of lipolysis.

Further, at the time of filing, there was evidence demonstrating that inhibition of lipolysis in adipose tissue is tied to the treatment of metabolic-related disorders such as dyslipidemia and atherosclerosis (see Lorenzen, et al., "Characterization of a G Protein-Coupled Receptor Nicotinic Acid", Molecular Pharmacology, 59:349-357 (2001) (cited in the supplemental IDS)). Further, elevated levels of adiponectin can significantly suppress the formation of atherosclerotic lesions in mice. cAMP, in turn, down-regulates adiponectin secretion, thereby leading to lower levels of adiponectin (see Delaporte, et al., "Pre- and post-translational negative effect of β-adrenoceptor agonists on adiponectin secretion: *in vivo* and *in vitro* studies", *Biochem. J.* 367:677-685 (2002) (enclosed and cited in the IDS of Jan. 8, 2008); Okamoto, "Adiponectin reduces atherosclerosis in apolipoprotein e-deficient mice", *Circulation*, 106:2767-2770 (2002) (cited in the supplemental IDS); and Matsuda, "Role of adiponectin in preventing vascular stenosis: the missing link of adipo-vascular axis", *J. Biol. Chem.*, 277(40):37487-37491 (2002) (enclosed and cited in the IDS of Jan. 8, 2008) for support that (a) cAMP is a down-regulator of adiponectin secretion; and (b) elevated levels of adiponectin significantly suppress the formation of atherosclerotic lesions in mice, while reduced levels result in augmented intimal proliferation in the vascular walls of adiponectin-null mice). Hence, these references demonstrate that there is tie between a decrease in lipolysis and cAMP levels and the treatment of metabolic-related disorders.

As the working examples show that compounds of the examples can decrease lipolysis and reduce the elevation of cAMP levels and the art, in turn, shows a tie between a decrease in these levels and the treatment of metabolic-related disorders, one of skill in art would accept that compounds of the present application would be useful to treat metabolic-related disorders such as dyslipidemia and atherosclerosis. Accordingly, one of skill in the art would be able to use the claimed methods without engaging in undue experimentation. For all of these reasons, Applicants respectfully assert that all of the requirements of 35 U.S.C. § 112, first paragraph, have been met and request that the claim rejections be withdrawn.

B. Solvates and Hydrates

Claims 1, 2, 12, 13 and 28 are rejected under 35 U.S.C. § 112, first paragraph, as allegedly failing to comply with the enablement requirement. The Office alleges that the specification "does not reasonably provide enablement for solvates or hydrates of [the claimed] compounds" (Office Action, page 7). Citing to *Morton International Inc. v. Cardinal Chemical Co.*, 28 U.S.P.Q.2d 1190 (Fed. Cir. 1993), the Offices states that "[t]here is no evidence that solvates or hydrates of the instantly claimed compounds exist" because "[i]f they did, they would have been formed" (Office Action, page 8). The Office further states that "[i]t is not the norm that one can predict with any accuracy a particular solvate form of an active compound will be more soluble, more easily handled in formulations or more bioavailable without actual testing *in vivo*" (Office Action, page 8). Because allegedly an "extremely large number of solvates and hydrates that could be encompassed by the claims", the Office states that "nothing short of extensive testing (none identified) would be needed to determine if additional derivatives exist and thus, such as scope as literally claimed herein is non-enabled" (Office Action, page 10).

As a preliminary matter, the Office has stated that "[i]t is not the norm that one can predict with any accuracy a particular solvate form of an active compound will be more soluble, more easily handled in formulations or more bioavailable without actual testing *in vivo*" (Office Action, page 8). Applicants respectfully note, however, that compliance with § 112, first paragraph, does not require that the solvates or hydrates be **more** bioavailable or **more** easily handled than the compounds of Formula I. Rather, it is sufficient to show that the solvates and hydrates can be made and used without undue experimentation.

Further, the Office's reliance on *Morton* is misplaced. In *Morton*, the claims were directed to organotin compounds having "partial connectivity". *Morton*, 28 U.S.P.Q.2d at 1193. Noting that "[e]ven with the aid of sophisticated analytical instrumentation and the use of model systems", there was no evidence that the claimed compounds with the required connectivity could even exist. *Id.* Further, there was no evidence the procedures in the specification or the defendant's process would produce compounds with the "partial connectivity". *Id.* at 1193-94. Applicants respectfully assert that the claimed solvates and hydrates present a far different situation from that in *Morton*. As summarized below, there is clear evidence that hydrates and

solvates are quite common and can be formed by routine methods. Hence, there is no question that hydrates and solvates can exist, unlike the compounds having partial connectivity in *Morton*. Further, unlike the unsuccessful preparative routes in *Morton*, the Office has failed to point to any section of the specification which suggests that Applicants attempted and failed to produce a solvate or hydrate of the claimed compounds.

Moreover, Applicants respectfully assert that the Office has not carried its burden to provide evidence or reasoning showing a sufficient reason to doubt that one of skill in the art could make the hydrates and solvates of the claimed compounds without undue experimentation. As will be appreciated, the test for whether experimentation would be undue is not merely quantitative since a considerable amount of experimentation is permissible, if it is merely routine. *Wands*, 8 U.S.P.Q.2d at 1404. In *Wands*, the Office had rejected the appealed claims, directed to methods for assaying HBsAg using high-affinity IgM monoclonal antibodies, as lacking enablement. *Id.* at 1402. The Office alleged that the production of high-affinity IgM anti-HBsAg antibodies was unpredictable and unreliable and, therefore, would require undue experimentation. *Id.* The Federal Circuit disagreed, finding that undue experimentation would not be required. *Id.* at 1406. Even though screening for hybridomas involved several, labor-intensive steps (see the steps in Table 1), the court found that this amount of effort was not excessive or undue, as the methods needed to practice the invention were well-known and the level of skill in the art was high. *Id.* The court noted that a finding of undue experimentation would not be required even if the success rate for producing the antibodies was only 2.8% as suggested by the Office (as contrasted with the 44% success rate advanced by the applicant). *Id.*

In stark contrast with the antibody-making procedures at issue in *Wands*, the preparation of hydrates and solvates of a particular organic molecule is a substantially easier and overwhelmingly simpler process, which requires significantly fewer steps and much less time than the preparation of a monoclonal antibody. Table 1 provides a step-by-step comparison of some of the major steps involved in the production of a monoclonal antibody (as disclosed in *Wands*) and the one step involved in making a hydrate or solvate. To make hydrates and

solvates, samples of the organic compound are exposed to water or various different solvents.[2] Once the hydrates and solvates are formed, they can be readily analyzed by routine methods or other routine techniques to detect and quantify the presence of hydrate or solvate molecules in the sample. Exposure of the organic compounds to water and various solvents is conducted through simple and routine methods such as letting the samples sit open to air for set amounts of time, as well as slurrying and/or crystallizing the samples from water or solvent. In fact, it is difficult to conceive of a scientific method that is simpler to perform than placing a powder on a dish and letting it sit out on a humid day. Other typical procedures for making and identifying hydrates and solvates are described in Guillory, "Generation of Polymorphs, Hydrates, Solvates, and Amorphous Solids", in Polymorphism in Pharmaceutical Solids, ed. Harry G. Brittain, vol. 95, chapter 5, Marcel Dekker, Inc., New York 1999, pages 183-226 (hereinafter "Guillory") (cited in the supplemental IDS). Hence, screening for hydrates and solvates merely uses methods that are very well known in the art and considered quite simple.[3] As is clearly shown in Table 1 and summarized above, the production of a monoclonal antibody is much more complex and time-consuming than the production of a hydrate or solvate, yet the *Wands* court concluded that the production of a monoclonal antibody was not excessive and undue. Hence, it is clearly inconsistent to allege that the production of hydrates and solvates would require undue

---

[2] For example, Guillory, "Generation of Polymorphs, Hydrates, Solvates, and Amorphous Solids", in Polymorphism in Pharmaceutical Solids, ed. Harry G. Brittain, vol. 95, chapter 5, Marcel Dekker, Inc., New York 1999, pages 183-226 (hereinafter "Guillory") at pages 202-205 and pages 205-208 describe the routine preparation of hydrates and solvates of compounds, respectively, as illustrated in the excerpts below:

> Simply exposing an anhydrous powder to high relative humidity can often lead to formation of a hydrate.

Guillory, page 204.

> Often, when solvents are employed in the purification of new drug substances by recrystallization, it is observed that the isolated crystals include solvent molecules...

Guillory, page 205.

[3] In fact, there are numerous companies that routinely provide this screening service (usually combined with polymorph screens) and advertise how quickly and efficiently they can identify hydrates and solvates. Example companies offering these services include Wilmington PharmaTech (Wilmington, DE) and Avantium Technologies (Amsterdam).

experimentation, while the production of monoclonal antibodies would not require undue experimentation.

The Office attempts to base its enablement rejection on unpredictability of solvate formation and (2) lack of working examples. Unpredictability was a major reason for the Office's rejection of the claims in *Wands*, yet the rejection was reversed by the Federal Circuit because, in part, all the methods needed to practice the invention were well-known and the level in the art was high. Accordingly, any unpredictability associated with hydrate or solvate formation that might exist is clearly outweighed by the fact that preparing and screening for hydrates and solvates is routine and employs well-known methods. With respect to lack of working examples, the courts have held that there is no requirement for a "working" example if the disclosure is such that one skilled in the art can practice the claimed invention. *In re Borkowski,* 164 U.S.P.Q. 642 (C.C.P.A. 1970); *Ex parte Nardi*, 229 U.S.P.Q. 79 (Pat. Off. Bd. App. 1986). Given that one skilled in the art could make and identify various hydrates and solvates of a particular organic molecule using the routine screening methods discussed above, no working example is necessary to enable the invention.

Further, after searching the PTO database of issued patents in a cursory manner, the following U.S. Patents were readily identified as having claims including hydrates and/or solvates, yet having no enablement rejections to the same: U.S. Pat. Nos. 7232823, 7230024, 7229991, 7211591, 7173037, 7157466, and 7105523. Applicants see no difference between these patents and the present application with respect to enablement of hydrates and solvates and, thus, believe that the enablement rejection in this application should be withdrawn. For all of these reasons, Applicants respectfully assert that all of the requirements of 35 U.S.C. § 112, first paragraph, have been met and request that the claim rejections be withdrawn.

Table 1

Step	Monoclonal Antibody	Hydrate or Solvate
1	immunize animal	expose the compound to water or solvent

Step	Monoclonal Antibody	Hydrate or Solvate
2	remove the spleen from the immunized animal	
3	separate the lymphocytes from the other spleen cells	
4	mix the lymphocytes with myeloma cells	
5	treat the mixture to cause fusion between the lymphocytes and the myeloma cells to make hybridomas that hopefully secrete the desired antibody	
6	separate the hybridoma cells from the unfused lymphocytes and myeloma cells by culturing in a medium in which only hybridoma cells survive	
7	culture single hybridoma cells (often 100 of different cells) in separate chambers	
8	assay the antibody secreted from each hybridoma culture to determine if it binds to the antigen	

III. The Claims Have Written Description

Claim 14 is rejected under 35 U.S.C. § 112, first paragraph, as allegedly failing to comply with the written description requirement. In particular, the Office alleges that the terms "α-glucosidase inhibitor, aldose reductase inhibitor, biguanide, HMG-CoA reductase inhibitor, squalene synthesis inhibitor, fibrate, LDL catabolism enhancer, angiotensin converting enzyme inhibitor, insulin secretion enhancer and thiazolidinedione" are not "defined in the specification so as to know the structures of the compositions that are included and/or excluded by the term" (Office Action, pages 10-11). Therefore, the Office asserts that claim 14 lacks adequate support.

Applicants respectfully note that the Office appears to be using the legal standard for definiteness, rather than legal standard for written description. As noted by the Federal Circuit, the "requirements of adequate description and definite claim, though closely intertwined, are analytically distinct." *F. Rengo Co. Ltd. et al. v. Molins Machine Company, Inc.*, 211 U.S.P.Q.

303, 320-21 (Fed. Cir. 1981). The purpose of the definiteness requirement is to "demarcate the boundaries of the purported invention, in order to provide notice to others of the limits 'beyond which experimentation and invention are undertaken at the risk of infringement'. *Id.* at 321. By contrast, the purpose of the written description requirement is that the specification describe the invention clearly enough so as to reasonably convey to a person of ordinary skill in the art that, as of the filing date sought, the inventor was in possession of the invention. *University of Rochester v. G.D. Searle & Co.*, 69 U.S.P.Q.2d 1886, 1894 (Fed. Cir. 2004). Accordingly, the proper inquiry is whether the specification describes the additional agents in claim 14 in a manner so as to reasonably convey to a person of ordinary skill in the art that Applicants were in possession of the invention, not whether one of ordinary skill in the art would know what compositions are included or excluded by the terms.[4]

As will be appreciated, it is "unnecessary to spell out every detail of an invention" as long as enough is included to convince a person of skill in the art that the inventor possessed the invention. *See Falkner v. Inglis*, 79 U.S.P.Q.2d 1001, 1007 (Fed. Cir. 2006). This is because "the patent specification is written for a person of skill in the art, and such a person comes to the patent with the knowledge of what has come before." *Id.*

Applicants note that claim 14 is directly analogous to the facts of *In re Herschler*, 200 U.S.P.Q. 711 (C.C.P.A. 1979), discussed by the Federal Circuit in *University of Rochester* case. In *Herschler*, the court found the term "steroidal agent" to have written description support in a claim reciting a method of enhancing skin penetration, involving topical administration of "an amount of a steroidal agent effective to produce the desired physiological effect" and "an amount of DMSO sufficient to effectively enhance penetration of said steroidal agent to achieve the desired physiological effect". *Herschler*, 200 U.S.P.Q. at 712. The *Rochester* court distinguished the facts in *Herschler* from the "non-steroidal compound that selectively inhibits activity of the PGHS-2 gene product" in *Rochester*. *Rochester*, 69 U.S.P.Q.2d at 1896. First, the court noted that there were several examples of physiologically active steroidal agents known to a person of ordinary skill in the art in *Herschler*, while there were no known non-steroidal agents

[4] Applicants note that they have addressed the separate definiteness rejection in section IV below.

with the required activity in *Rochester*. *Id.* Second, the court distinguished claims where the functionally described element is the novel element and those where the functionally described element is not the novel element. *Id.* In particular, the court noted that DMSO was the novel element in the *Herschler* claim, not the physiologically active steroidal compound. *Id.* Significantly, the court noted that "a different question would have been posed if the claim in *Herschler* had been drawn to novel steroidal agents rather than a method of increasing penetration of these agents using DMSO." *Id.* Accordingly, patent applicants have "some flexibility in the 'mode selected for compliance'" with the written description requirement, where the novel element of the claims is other than the functionally described agent and wherein there are examples of the agent known to one of ordinary skill in the art. *Id.*

Claim 14 recites a " pharmaceutical composition according to claim 13 **further comprising an agent** selected from the group consisting of α-glucosidase inhibitor, aldose reductase inhibitor, biguanide, HMG-CoA reductase inhibitor, squalene synthesis inhibitor, fibrate, LDL catabolism enhancer, angiotensin converting enzyme inhibitor, insulin secretion enhancer and thiazolidinedione." Hence, similar to the DMSO in the *Herschler* claim, the point of novelty is not the agent of claim 14, but rather the compound of Formula I recited in claim 13. Moreover, the specification recites examples for each of the agents recited by claim 14 (see specification at page 39, line 32, through page 41, line 25). Accordingly, Applicants respectfully assert that the specification describes the claimed invention in a way that shows that Applicants were in possession of the claimed invention. For all of these reasons, Applicants respectfully assert that all of the requirements of 35 U.S.C. § 112, first paragraph, have been met and request that the rejection of claim 14 be withdrawn.

IV. The Claims Are Definite

Claim 14 is rejected under 35 U.S.C. § 112, second paragraph, as being allegedly indefinite as to the term "α-glucosidase inhibitor". In particular, the Office alleges that "[i]t is not possible that all current and potential α-glucosidase inhibitors can be agents for the instantly claimed composition" (Office Action, page 11). Further, the Office asserts that a "claim is

indefinite where it merely recites a use without any active, positive steps delimiting how this use is actually practiced" (Office Action, page 11).

As will be appreciated, a claim should be deemed to be indefinite only "when a claim remains insolubly ambiguous without a discernible meaning after all reasonable attempts at construction." *Metabolite Labs., Inc. v. Lab. Corp. of Am. Holdings*, 71 U.S.P.Q.2d 1081, 1089 (Fed. Cir. 2004). Further, the Applicant is entitled to act as his or her own lexicographer in defining claim terms. M.P.E.P. § 2111.01. As such, "claims need not 'be plain on their face in order to avoid condemnation for indefiniteness; rather, what [the Office is] asked is [whether] the claims [are] amenable to construction.'" *SmithKline v. Apotex*, 403 F.3d 1331, 1340 (Fed. Cir. 2005), citing *Exxon Research & Engineering Corp. v. United States*, 265 F.3d 1371, 1375 (Fed. Cir. 2001).

Given these principles, Applicants respectfully assert that claim 14 does not lack discernible meaning to one of skill in the art, in light of the teachings of the specification. First, Applicants note that the specification clearly defines what is meant by the term "α-glucosidase inhibitor" at page 39, line 32, through page 40, line 2, as well as what is meant by the remaining terms in claim 14 (see specification at page 40, line 3, through page 41, line 25). Accordingly, the term "α-glucosidase inhibitor" in claim 14 is not "insolubly ambiguous without a discernable meaning after all reasonable attempts at construction". As to the Office's statement that "[i]t is not possible that all current and potential α-glucosidase inhibitors can be agents for the instantly claimed composition", Applicants respectfully assert that the Office has not provided any reasoning for this conclusory opinion. Finally, the Office's assertion that "claim is indefinite where it merely recites a use without any active, positive steps delimiting how this use is actually practiced" seems apposite to claim 14 which recites a pharmaceutical composition rather than a method requiring positive steps. For all of these reasons, Applicants respectfully assert that all of the requirements of 35 U.S.C. § 112, second paragraph, have been met and request that the rejection of claim 14 be withdrawn.

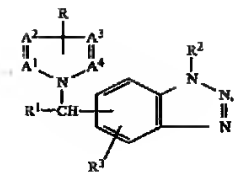
V. The Claims Are Novel

Claims 1, 2, 4-8, 12, 13, and 18 are rejected under 35 U.S.C. § 102(a) as being unpatentable over U.S. Patent 4,943,574 (hereinafter "the '574 patent"). The Office correctly states that the '574 patent discloses 1-butyl-1H-benzotriazole-5-carboxylic acid in Example 2. However, Applicants respectfully direct the Office's attention to proviso e) of claim 1 which states:

> **when R_2, R_3, R_4 and R_5 are all H then R_1 is not** 2-amino-2-carboxy-ethyl, pyrrolidin-1-ylmethyl, isopropyl, methyl, benzyl, **n-butyl**, or carboxymethyl

(emphasis added). When R_2, R_3, R_4 and R_5 are all H as in the 1-butyl-1H-benzotriazole-5-carboxylic acid compound, proviso e) explicitly does not allow the 1-position, or R_1, to be butyl. Hence, Applicants respectfully assert that this species does not fall within the scope of claim 1 and, therefore, cannot destroy the novelty of claim 1. Further, Applicants have deleted this species from claim 12, in order to preserve antecedent basis from claim 1. Moreover, Applicants have been unable to locate any portion of the '574 patent which discloses a species falling within the scope of claim 1. Accordingly, Applicants respectfully assert that claim 1, and dependent claims thereof, are novel over the '574 patent.

As to claim 13, Applicants note that claim 13 recites a pharmaceutical composition. By contrast, the 1-butyl-1H-benzotriazole-5-carboxylic acid compound in Example 2 of the '547 patent is disclosed merely as an intermediate in the synthesis of the final active compounds as it is missing the $A^1A^2A^3A^4N$ ring of Formula I of the '574 genus (shown below). As such, the '547 patent fails to disclose a pharmaceutical composition containing the 1-butyl-1H-benzotriazole-5-carboxylic acid intermediate. Moreover, Applicants have been unable to locate any portion of the '574 patent which discloses a pharmaceutical composition falling within the scope of claim 13. Accordingly, Applicants respectfully assert that claim 13, and dependent claims thereof, are novel over the '547 patent. For all of these reasons, Applicant respectfully asserts that all of the requirements of 35 U.S.C. § 102 have been met and request that the claim rejections be withdrawn.



VI. The Claims Are Non-Obvious

Claims 1-14 and 28 are rejected under 35 U.S.C. § 103(a) as being unpatentable over the '574 patent. The Federal Circuit has recently emphasized that the Supreme Court's decision in *KSR Int'l Co. v. Teleflex Inc.*, 127 S. Ct. 1727, 82 U.S.P.Q.2d 1385 (2007) relied on three assumptions in determining the obviousness of claimed subject matter over the prior art:

> First, KSR assumes a **starting reference point or points in the art**, prior to the time of invention, from which a skilled artisan might identify a problem and pursue potential solutions. Second, KSR presupposes that the record up to the time of invention would give **some reasons, available within the knowledge of one of skill in the art, to make particular modifications to achieve the claimed compound.** See Takeda, 492 F.3d at 1357 ("Thus, in cases involving new chemical compounds, **it remains necessary to identify some reason that would have led a chemist to modify a known compound in a particular manner to establish *prima facie* obviousness of a new claimed compound.**"). Third, the Supreme Court's analysis in KSR presumes that the record before the time of invention would supply some reasons for narrowing the prior art universe to a "finite number of identified, predictable solutions," 127 S. Ct. at 1742.

Eisai Co. Ltd. v. Dr. Reddy's Laboratories Ltd., 87 U.S.P.Q.2d 1452, 1456-1457 (Fed. Cir. 2008) (emphasis added). Accordingly, in establishing a *prima facie* case of obviousness for a chemical compound, the Office bears the burden of showing: (1) a reasoned identification of a lead compound; and (2) some reason that would have led a chemist to modify the lead compound in a particular manner.

With respect to intermediate compounds in prior art synthetic routes, the Federal Circuit has made it clear that the reason for modifying the prior art intermediate compound does not arise merely because the final product in the synthetic route has a disclosed utility. *In re Lalu*, 223 U.S.P.Q. 1257 (Fed. Cir. 1984). For example, in *Lalu*, the claimed fluorinated sulfonyl chloride compounds were structurally similar to prior art intermediate compounds used to form

fluorinated sulfonic acids which had utility as neutralizing agents, alkylation catalysts, metal cleaners, and high energy fuels. *Id.* at 1257-58. In reversing the Board's finding of obviousness based on the structural similarity of the intermediate and claimed compounds, the Federal Circuit reiterated that a *prima facie* case of obviousness cannot be premised on mere structural similarity when there is no utility disclosed for the prior art compound, as was the case for the intermediate sulfonyl chlorides in *Lalu*. *Id.* at 1259-60. Further, the fact that the end products of the synthesis had utility was insufficient basis for a *prima facie* case of obviousness. *Id.* Instead, the court emphasized that there must be a reason to stop the synthesis at the intermediate stage, isolate the intermediate compound, and test it for certain properties with a reasonable expectation of producing an active compound:

> The PTO places great emphasis on the label "useful", contending that because the Oesterling final product is "useful", the intermediate sulfonyl chlorides are also "useful". That there is no common-properties presumption accorded to an intermediate and the end product of the reaction involving that intermediate necessarily means that there is no presumption that an intermediate's utility would be the same as that of the end product...There is no disclosure that the Oesterling compounds would have any properties in common with those of appellants' compounds, as those properties of the former relate to the use of the compounds for base neutralization, catalysis, metal cleaning, and fuel. The mere fact that Oesterling's sulfonyl chlorides can be used as intermediates in the production of the corresponding sulfonic acids does not provide adequate motivation for one of ordinary skill in the art to stop the Oesterling synthesis and investigate the intermediate sulfonyl chlorides with an expectation of arriving at appellants' claimed sulfonyl halides for use as corrosion inhibiting agents, surface active agents, or leveling agents.

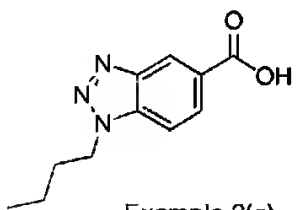
Id.

The Federal Circuit recently applied this reasoning regarding intermediate compounds in a post-*KSR* case, *Ortho-McNeil Pharmaceutical Inc. v. Mylan Laboratories Inc.*, 86 U.S.P.Q.2d 1196, 1120 (Fed. Cir. 2008). In *Ortho-McNeil*, the inventor had discovered the epilepsy drug, topiramate, by testing a reaction intermediate in the synthesis of potential diabetes drugs. *Id.* at 1198. The court concluded that a *prima facie* case of obviousness had not been shown, applying reasoning similar to that in *Lalu*:

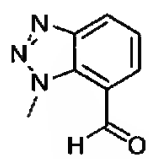
> Moreover this invention, contrary to Mylan's characterization, does not present a finite (and small in the context of the art) number of options easily traversed to show obviousness. In this case, the record shows that a person of ordinary skill would not even be likely to start with 2,3:4,5-di-isopropylidene fructose (DPF), as Dr. Maryanoff did. Beyond that step, however, the ordinarily skilled artisan would have to have some reason to select (among several unpredictable alternatives) the exact route that produced topiramate as an intermediate. Even beyond that, the ordinary artisan in this field would have had to (at the time of invention without any clue of potential utility of topiramate) stop at that intermediate and test it for properties far afield from the purpose for the development in the first place (epilepsy rather than diabetes). In sum, this clearly is not the easily traversed, small and finite number of alternatives that *KSR* suggested might support an inference of obviousness.

Id. at 1201.

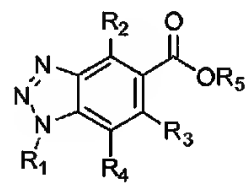
Applicants respectfully assert that the claims are non-obvious over the '574 patent. The Office points to 1-butyl-1H-benzotraizole-5-carboxylic acid in Example 2(c) (hereinafter "Example 2(c)") and 1-methyl-1H-benzotriazole-7-carboxaldehyde in Example 3(a) (hereinafter "Example 3(a)"), which the Office alleges anticipate claim 1 (Office Action, pages 13-14; see the structures of Example 2(c) and 3(a) below, along with the structure of Formula I of claim 1). The Office further alleges that these compounds are "used pharmaceutically to treat disorders ranging from estrogen to thomboxane synthetase disorders" (Office Action, page 13). The Office concludes that because "the art teaches the process of making a species claimed in the instant application", "[o]ne of ordinary skill in the art would be able to optimize the reaction conditions to include other more generic compounds based on [its] teachings" (Office Action, page 14).



Example 2(c)



Example 3(a)



(I)

At the onset, Applicants respectfully note neither Example 2(c) or 3(a) anticipate independent claim 1 or 13, or dependent claims thereof. Example 3(a) is clearly missing the

carboxylic acid or ester at the 5-position of the benzotriazole ring as shown in Formula I of claims 1 and 13, shown above. Further, Example 2(c) is excluded by proviso e) of claim 1 as discussed in section V of this response. In addition, Example 2(c) does not anticipate claim 13, or dependent claims thereof, as the '574 patent fails to disclose the use of Example 2(c) in a pharmaceutical composition. Accordingly, the Office's reasoning regarding Examples 2(c) and 3(a) is misplaced. As such, Applicants note that the Office has failed to provide any reasoned identification of a lead compound or a reason for making the specific molecular modifications to the lead compound as required by the *Eisai* court. Accordingly, for this reason alone, the Office has not carried its burden to establish a *prima facie* case of obviousness.

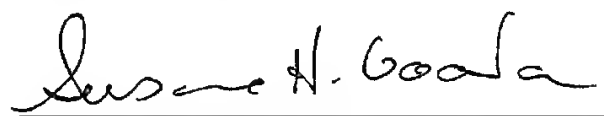
Moreover, Applicants respectfully note that Examples 2(c) and 3(a) of the '574 patent are intermediate compounds, as they are missing the $A^1A^2A^3A^4N$ ring of Formula I of the '574 genus (shown in section V of this response). As such, the Office is simply mistaken that Examples 2(c) and 3(a) can be "used pharmaceutically to treat disorders ranging from estrogen to thomboxane synthetase disorders" (Office Action, page 13). Hence, the '574 patent fails to disclose any utility for Examples 2(c) and 3(a). As explained in *Lalu*, the fact that the end products of the '574 patent have utility does not support a *prima facie* case of obviousness based on the intermediate compounds in the absence of a reason to stop the synthesis at the intermediate stage, isolate the intermediate compound, and test it for certain properties with a reasonable expectation of producing an active compound. As the '574 patent is silent as to any such reasoning, Applicants respectfully assert that the Office has failed to establish a *prima facie* case of obviousness based on Examples 2(c) and 3(a) of the '574 patent. In addition, Applicants respectfully note that there is no overlap between the Formula I genus of the '574 patent and that of claim 1 or 13 of the present application. For all of these reasons, Applicant respectfully asserts that all of the requirements of 35 U.S.C. § 103(a) have been met and request that the claim rejections be withdrawn.

VII. Conclusion

Applicants respectfully assert that rejections of record have been overcome by way of this response. Allowance of all claims is respectfully requested. The Examiner is urged to contact Applicant's undersigned representative at (302) 778-8411 if there are any questions regarding the claimed invention.

The Commissioner is hereby authorized to debit any fee due or credit any overpayment to Deposit Account No. 06-1050. Further, if not accompanied by an independent petition, this paper constitutes a Petition for an Extension of Time for an amount of time sufficient to extend the deadline if necessary and authorizes the Commissioner to debit the petition fee and any other fees or credit any overpayment to Deposit Account No. 06-1050.

Respectfully submitted,



Susanne H. Goodson, Ph.D.
Reg. No. 58,450

Date: December 19, 2008

Fish & Richardson P.C.
P.O. Box 1022
Minneapolis, MN 55440-1022
Telephone: (302) 778-8411
Facsimile: (877) 769-7945

Enclosures: Matsuda and Delporte references

Pre- and post-translational negative effect of β-adrenoceptor agonists on adiponectin secretion: *in vitro* and *in vivo* studies

Marie-Laure DELPORTE*, Tohru FUNAHASHI†, Masahiko TAKAHASHI†, Yuji MATSUZAWA† and Sonia M. BRICHARD*[1]

*Unit of Endocrinology and Metabolism, University of Louvain, Faculty of Medicine, UCL 5530 Avenue Hippocrate, 55, B-1200 Brussels, Belgium, and †Department of Internal Medicine and Molecular Science, Graduate School of Medicine, Osaka University, Osaka 565-0871, Japan

The adipose-derived hormone, adiponectin (ApN), has a role in fuel homoeostasis, insulin action and atherosclerosis. Regulation of ApN by catecholamines has scarcely been investigated. We examined the effects of β-adrenergic agonists (and their second messenger, cAMP) on ApN gene expression, production and secretion in mouse *in vitro* and *in vivo*; their effects in human fat were also briefly studied *in vitro*. β-Adrenergic agonists and cAMP inhibited ApN gene expression in human visceral adipose tissue. Likewise, cAMP down-regulated ApN mRNAs in cultured mouse explants from visceral and subcutaneous regions. The amount of ApN released into the medium decreased concomitantly. cAMP also caused qualitative changes in ApN secretion. Under basal conditions, ApN was secreted as a single 32 kDa species. In the presence of cAMP, an additional and probably immature (not modified post-translationally) 30 kDa species was also sorted. This altered secretion resulted from cAMP-induced quantitative and qualitative changes of ApN within the adipocyte. Under basal conditions, the 32 kDa form of ApN was mainly associated with high-density microsomes (HDMs), while the 30 kDa species was confined to a pool recovered with the cytosol fraction. cAMP depleted intracellular ApN at the expense of both HDM and cytosol fractions, and abnormally targeted ApN species to the different subcellular compartments as a result of impaired maturation. β-Adrenergic agonists mimicked the inhibitory effects of cAMP on ApN mRNA and secretion, the β_3-agonist BRL37344 being the most potent. Administration of BRL37344 to mice reduced ApN mRNAs in both adipose regions, and ApN levels in plasma. In conclusion, β-agonists inhibited ApN production and maturation, and thus exerted a dual (pre- and post-translational) negative effect on ApN secretion by cultured mouse adipose explants. ApN inhibition by β-agonists was reproduced in mouse *in vivo* and in humans *in vitro*. ApN down-regulation may have an important role in fuel homoeostasis, insulin resistance and stress-induced atherosclerosis.

Key words: adipocytes, atherosclerosis, insulin resistance, leptin, obesity.

INTRODUCTION

Adipose tissue secretes a large number of physiologically active peptides [1] that often share structural properties of cytokines, and are therefore referred to collectively as 'adipocytokines'. One of these, adiponectin (ApN), is expressed exclusively in differentiated adipocytes. This hormone is composed of an N-terminal collagenous domain and a C-terminal globular domain [2], and is secreted into the blood where its concentration is high. ApN increases muscle fatty acid oxidation and causes weight loss in mice [3]. It is also a potent insulin enhancer in mouse models of obesity, lipoatrophy and/or diabetes [4,5]. Eventually, ApN attenuates endothelial inflammatory responses and macrophage-into-foam cell transformation *in vitro*, thereby potentially preventing the development of atherosclerosis [6, 7]. Further support for the metabolic effects of ApN comes from clinical and genetic studies. Thus plasma ApN levels are decreased in human subjects with obesity [8], type 2 diabetes [9] or cardiovascular disease [6]. Recent genome-wide scans have mapped a susceptibility locus for type 2 diabetes and metabolic syndrome to the chromosome 3q27, where the ApN gene is located [10,11].

The mechanisms involved in the regulation of ApN have not been fully elucidated. Although catecholamines have a major role in fuel homoeostasis, in counteracting insulin action and in promoting stress-induced atherosclerosis, their influence on ApN production has scarcely been investigated. A single study has reported a reduction in ApN mRNA levels by isoproterenol in murine clonal 3T3-L1 pre-adipocytes differentiated *in vitro* [12]. However, ApN may be regulated at the post-translational level and these modifications may be a determinant for protein activity [2,13]. Moreover, pre-adipocyte differentiation in an *in vivo* context has been found to be a prerequisite for optimal adipocytokine expression and hormonal responsiveness [14]. Furthermore, the effects of catecholamines on circulating ApN levels have not been explored; their influence on human ApN is also currently unknown.

In the present study, we examined the effects of β-adrenergic agonists (and cAMP, their second messenger) on ApN gene expression, tissue content and distribution, and secretion in cultured mouse adipose tissue or mature adipocytes. We extended this work to mice treated with β-agonists *in vivo*. Lastly, some data on the influence of catecholamines on human fat have also been presented.

MATERIALS AND METHODS

Subjects

Visceral (omental) adipose tissue was obtained from nine subjects (three men and six women; age 48.1 ± 5.1 years; body mass

Abbreviations used: ApN, adiponectin; Cyt, cytosol; FCS, foetal calf serum; HDM, high-density microsome, LDM, low-density microsome, MEM, minimum essential medium; PM, plasma membrane; rApN, recombinant adiponectin; s.c. subcutaneous(ly), TM, total membrane, TNFα, tumour necrosis factor-α.

[1] To whom correspondence should be addressed (e-mail brichard@endo.ucl.ac.be).

index, 31.6 ± 2.3 kg/m²) undergoing elective abdominal surgery after an overnight fast. No patient had a history of diabetes or coronary heart disease, and none had undergone any significant weight change. Patients receiving medication known to influence adipose tissue mass or metabolism were excluded. The study was carried out in accordance with the Declaration of Helsinki of the World Medical Association, and had the approval of the local ethical committee.

Adipose biopsies were placed in PBS and transported to the laboratory within 5–10 min of sampling, and then were prepared for culture of explants. For each culture, adipose tissue from only one subject was used.

Animals

Female mice aged 11 weeks (strain C57BL/6J or NMRI) were purchased from IFFA Credo (Brussels, Belgium). The genetic background of mice did not influence the results, and data from both strains of mice were pooled for presentation. The animals received *ad libitum* a common laboratory chow (A04; Usine d'Alimentation Rationnelle, Villemoisson-Sur-Orge, France) and were housed at a constant temperature (22 °C) with a fixed 12 h light: 12 h dark cycle.

For *in vitro* studies (adipose tissue culture), mice were killed in the morning. Visceral (intraperitoneal–retrovesical) and subcutaneous (inguinal) fat pads were quickly removed under sterile conditions. It was necessary to pool tissues from several mice to obtain enough material of a given depot to allow direct comparisons to be made between different experimental conditions.

For the *in vivo* study, treatment with the β-adrenergic agonist began at 06:00 h. BRL37344 (Sigma-Aldrich, Bornem, Belgium) was dissolved in saline and used at a concentration of 0.11 mg/ml. This compound was injected subcutaneously (s.c.) to mice at a dose of 2 mg/kg of body weight, twice after 8 hourly intervals (i.e. at 06:00 h and 14:00 h). Control mice received the vehicle only. At the end of the experiment (i.e. 16 h after the first injection), visceral and inguinal fat pads were immediately removed, frozen in liquid nitrogen and stored at −70 °C; blood samples were also saved, and plasma was stored at −20 °C.

The University Animal Care Committee has approved all procedures.

Adipose tissue culture

Small fragments of human or mouse adipose tissue (2–3 mm³; explants) were prepared and cultured for up to 12 h (human) or 10 h (mouse) in 100 mm Petri dishes containing 10 ml of minimal essential medium (MEM) with Earle's salts supplemented with 10 % (v/v) foetal calf serum (human) or 0.5 % BSA (mouse) and antibiotics as described previously [15–17]; 600 mg (human) or 200 mg (mouse) of adipose tissue was cultured per dish. In some preliminary experiments with mouse explants, reducing the amount of tissue per dish (to 40 mg) did not affect ApN mRNA levels; likewise, renewing culture medium was without effect. Cell viability, as assessed by low release of lactate dehydrogenase and triacylglycerols into the medium under basal conditions [16], did not change over the course of the culture. Glucose concentrations in the medium also remained stable ([18]; results not shown).

Different agents [all from Sigma-Aldrich, except for dobutamine (Dobutrex®, Eli Lilly, Brussels, Belgium), fenoterol (Berotec®, Boehringer Ingelheim, Brussels, Belgium) and a polyclonal antibody directed against murine tumour necrosis factor-α (TNFα; R&D Systems Europe, Abingdon, U.K.)] were added to the medium in accordance with the experimental protocols. At the end of the culture, aliquots of medium were saved, and explants were rinsed in PBS, frozen in liquid nitrogen and stored at −70 °C.

RNA extraction and Northern blot analysis

Total RNA was extracted and subjected to Northern blot analysis [16]. The cDNA probe for mouse ApN was obtained after reverse transcription-PCR on total RNA from mouse adipose tissue (sense primer: 5′-TGGAGAGAAGGGAGAGAAA-3′ and antisense primer: 5′-AGAAAGCCAGTAAATGTAGAG-3′). The identity of the 528 bp product was confirmed by mapping with restriction endonucleases. The probes for human ApN and mouse cyclophilin have been described elsewhere [18,19]. After hybridization with the radiolabelled probes [18], the filters were exposed to autoradiographic films. Absorbances of the mRNA bands on the blots and of 18 S rRNA on the membranes were quantified by scanning densitometry (Image Master TotalLab, Amersham Biosciences). Levels of specific mRNA were expressed relative to those of 18 S rRNA. Internal standards (pooled RNA from 2–3 patients or from several mice) were always loaded on each gel to allow direct comparisons between different blots.

Preparation of subcellular fractions from adipose tissue or cells

Mouse explants were fractionated into total membranes (TMs, i.e. plasma and microsomal membranes) and cytosol (Cyt), as described by Le Marchand-Brustel et al. [20]. With this simple method, recovery of ApN in each fraction was complete (compare ApN levels in control homogenates with the sum of control levels in TM and Cyt; see Figure 3B). ApN concentrations in each fraction were not influenced by the use of fresh or previously frozen tissue that was homogenized in the original buffer [20], or in another, i.e. HES buffer (see below), with a motor-driven metal crusher (10 s) or a gentler Dounce homogenizer (10 strokes; results not shown).

In some experiments, we studied further ApN distribution within different membrane compartments in isolated mouse adipocytes. For each experimental condition, fat cells were prepared from 6 g of tissue (pooled dishes of cultured explants) as described previously [21]. Cells were rinsed twice in HES buffer [20 mM Hepes (pH 7.4)/250 mM sucrose/1 mM EDTA/1 mM PMSF/1 μM aprotinin], Dounce-homogenized and then fractionated into high-density microsomes (HDMs), low-density microsomes (LDMs), plasma membranes (PMs) and Cyt, according to slight modifications [22] of the protocol of Simpson et al. [23]. HDMs are enriched with endoplasmic reticulum and may also contain PM and LDM markers, whereas LDMs contain Golgi apparatus, *trans*-Golgi network and endosomes [24]. All fractions were stored at −70 °C. Protein concentrations were measured in each fraction by the Bradford method.

Quantification of ApN or leptin

Aliquots from subcellular adipose tissue or cell fractions (2.5–10 μg of protein), culture medium (30 μl) or serum (0.5 μl) were solubilized in modified Laemmli buffer, and then subjected to SDS/PAGE and immunoblotting using a monoclonal antibody directed against ApN [ANOC 9108 [25]; 1:6500 dilution in Tris-buffered saline with 0.05 % (v/v) Tween 20; [18]]. Although initially raised against human ApN, this antibody also recognizes mouse (and bovine) ApN due to high interspecies homology (see Figure 2F). After reaction with a secondary antibody (anti-mouse IgG–horseradish peroxidase), the blots were treated with enhanced chemiluminescence (ECL® Plus; Amersham Pharmacia) and subjected to autoradiography. Two forms of ApN were

detected on Western blots: one that migrates like recombinant ApN (rApN; Linco Research, St. Charles, MO, U.S.A.) as a 30 kDa band, and one that is found in plasma that migrates as a 32 kDa band (see Figure 2F). These signals could not be detected when blots were re-probed with an isotype-matched irrelevant antibody used instead of ANOC 9108 (results not shown). The absorbance of each ApN band was scanned individually using a densitometer as described previously. ApN concentrations in samples were then calculated from a linear standard curve generated by increasing amounts of mouse rApN (like that shown in Figure 2F). As rApN generates as a 30 kDa signal, it should, in theory only be used to quantify the 30 kDa species in samples. However, in practice, it was used as a standard for both forms of ApN. Because ANOC 9108 cross-reacted markedly with foetal calf serum (FCS), and human cultures were performed in the presence of FCS, ApN secretion could not be reliably quantified in human experiments and was measured in mice only.

Leptin levels were measured in adipocyte fractions by RIA, using a commercially available kit (RIA mouse leptin kit; Mediagnost, Reutlingen, Germany); samples (100 μl) were run in duplicate.

Presentation of the results

Results are the means ± S.E.M. for the indicated numbers of patients, mouse adipose tissue pools (*in vitro* experiments) or individual mice (*in vivo* study). Comparisons between two conditions were made using two-tailed unpaired or paired Student's *t* test, as appropriate. Comparisons of at least three conditions were carried out by ordinary or repeated ANOVA followed by the Newman–Keuls (comparisons of all pairs) or Dunnett's tests (all versus control) as appropriate. Differences were considered statistically significant at $P < 0.05$.

RESULTS

In human visceral adipose tissue, cAMP induced a 65% fall in the levels of ApN mRNA (approx. 4.5 kb transcript) after 12 h of culture. Isoproterenol, a pure β-adrenergic agonist, already inhibited ApN mRNA by approx. 30% after 8 h (Figure 1).

The effects of cAMP and, subsequently, of β-adrenoceptor activation were characterized further in 10 h cultured mouse adipose explants. In the basal state, the mouse ApN gene (approx. 1.3 kb transcript) was expressed more strongly in visceral than in inguinal (subcutaneous) fat, whereas cyclophilin mRNA and 18 S rRNA levels were similarly expressed (Figure 2A). The presence of cAMP for 10 h caused a 60–70% decrease in ApN mRNA in both depots, without affecting cyclophilin and 18 S parameters (Figure 2A).

The time course of the effects of cAMP on ApN gene expression in mouse explants is shown in Figures 2(B) and 2(C). Under basal (control) conditions, a spontaneous decrease of ApN mRNA occurred in both adipose regions (Figures 2B and 2C), whereas cyclophilin and 18 S parameters were again unchanged (results not shown). This decline of ApN mRNA was not due to some potential release of TNFα by explants, because it was unaffected by immunoneutralization of medium with large amounts of anti-TNFα antibody [26] or inhibition of TNFα production by pentoxifylline at a high concentration [26] (Table 1a, no change compared with controls after 10 h of culture). However, this decline was accelerated by cAMP and prevented by actinomycin D, an inhibitor of transcription (Figures 2B and 2C). The inhibitory effect of cAMP on ApN mRNA was detectable from 90 min in visceral fat (i.e. when basal ApN mRNAs were still stable), and 10 h in subcutaneous fat. This effect of cAMP was partly reversed in the presence of actinomycin D (tested only in visceral fat; Figure 2B) and could not be explained by enhanced lipolysis, since saturated or unsaturated fatty acids did not influence basal ApN gene expression (Table 1b).

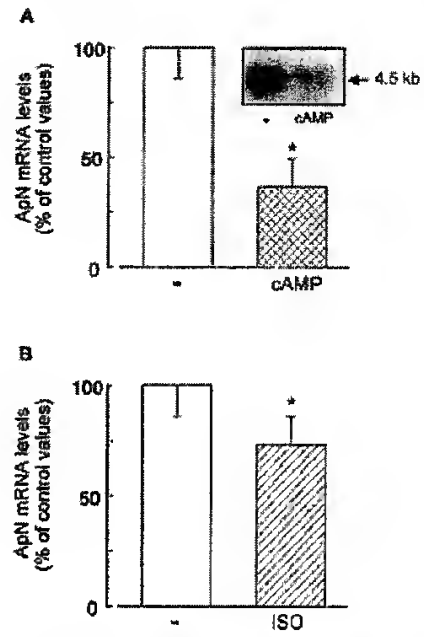


Figure 1 Inhibitory effect of cAMP (A) or isoproterenol (B) on ApN gene expression in human visceral adipose tissue

Explants were cultured in MEM with or without 1 mM dibutyryl-cAMP (cAMP) for 12 h or 10 μM isoproterenol (ISO) for 8 h. mRNA levels were expressed as percentages of control values (−, i.e. obtained in MEM not supplemented with hormones or agents). Values are the means ± S.E.M. for 4 (cAMP) or 5 (ISO) subjects. *$P < 0.05$ for the effect of the test agent

Table 1 Effect of inhibiting TNFα action/production (a), and of non-esterified fatty acids (b), on ApN mRNA levels in cultured mouse adipose tissue from visceral and inguinal regions

Tissues from both regions were simultaneously sampled in mice and explants were cultured for 10 h in MEM without (controls) or with the indicated agents. TNFα in medium was immunoneutralized with large amounts of anti-TNFα antibody or TNFα production was inhibited by a high concentration of pentoxifylline as previously described [26]. mRNA levels were expressed as percentages of respective control values obtained in visceral inguinal explants after 10 h of culture. Results are the means ± S.E.M. for four pools of adipose tissue, each composed of five mice. N.D., not done. Differences versus respective controls, not significant.

	ApN mRNA levels (% of 10 h control values)	
	Visceral	Inguinal
(a) Inhibition of TNFα action or production		
Anti-TNFα antibody (4 μg/10 ml)	90 ± 12	119 ± 14
Pentoxifylline (1 mM)	80 ± 9	72 ± 8
(b) Non-esterified fatty acids		
Oleic acid (375 μM)	105 ± 11	N.D.
Oleic acid (500 μM)	114 ± 14	N.D.
Palmitic acid (500 μM)	101 ± 14	N.D.

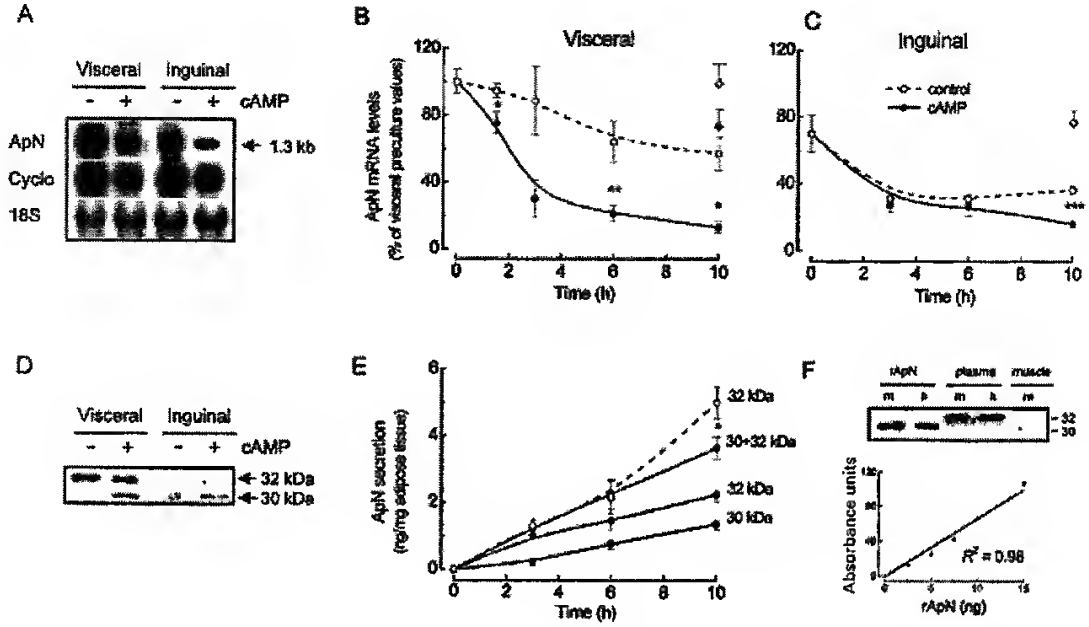


Figure 2 Effect of cAMP on ApN gene expression and protein secretion from mouse visceral and inguinal adipose tissues

Tissues from both regions were simultaneously sampled in mice, and explants were cultured for up to 10 h in MEM with or without 1 mM cAMP. **(A)** Representative Northern blot of depot-specific ApN mRNA abundance after 10 h of culture in control conditions (−), and inhibition of ApN mRNA by 10 h cAMP (+) in both depots. Cyclophilin mRNA and 18 S rRNA levels are shown for comparison. **(B, C)** Time course of the effects of cAMP on ApN gene expression in visceral and inguinal explants cultured for up to 10 h without (○) or with (●) cAMP. In some experiments, cultures were performed with 2 μM actinomycin D in the absence (◇) or in the presence (◆) of cAMP for 10 h. mRNA levels were expressed as percentages of pre-culture values (i.e. zero time) measured in visceral fat. **(D)** Western blot of ApN secreted in medium by explants from both regions cultured for 10 h without (−) or with (+) cAMP. Equal volumes (30 μl) of culture medium were loaded on each lane. **(E)** Quantification of ApN secreted by visceral explants over the course of the culture. Each ApN species (30 kDa or 32 kDa band) was scanned individually and quantified by using mouse rApN as standards. ApN released in medium was expressed as ng per mg of adipose tissue. **(F)** Representative standard curve obtained with increasing amounts of mouse rApN. The inset shows the primary antibody used for immunoblotting cross-reacts with mouse (m) or human (h) recombinant (30 kDa) or plasma (32 kDa) ApN, while no signals could be detected in mouse skeletal muscle. Results presented in panels **B**, **C** and **E** are the means ± S.E.M. for six (three for actinomycin D experiment) pools of adipose tissue, each composed of ten mice. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ for the effect of cAMP versus respective controls (i.e. ○).

In agreement with the depot-specific pattern of mRNA expression, the amount of ApN secreted into the medium by visceral fat exceeded that by inguinal fat (Figure 2D). Regional differences in secretion have already been reported for other adipocytokines [1]. Under our experimental conditions, release of ApN by the inguinal depot was so low that it could not be reliably quantified; this study will therefore focus on ApN secretion by visceral fat. In basal medium, ApN was secreted as a single 32 kDa species that increased in a time-dependent manner. In the presence of cAMP, an additional 30 kDa species was released, and the total amount of ApN (32 + 30 kDa) secreted over 10 h was lower than that accumulated under control conditions (Figure 2E). It is noteworthy that recombinant ApN migrates as a 30 kDa species (Figure 2F). This suggests that the 30 kDa species recovered in the medium may be an immature form of ApN that did not become modified post-translationally.

To examine the influence of cAMP on the different forms of ApN in crude adipose tissue fractions, explants were separated into TMs and Cyt. The 30 kDa species of ApN was recovered in adipose tissue homogenates and Cyt, while the 32 kDa species was present mainly in TMs (Figure 3A). However, when large amounts of protein were loaded, a faint 32 kDa signal was also observed in the homogenate (results not shown). After 10 h of culture in basal medium, ApN levels (in ng/μg of protein) were distributed equally between TM and Cyt fractions. cAMP markedly decreased ApN concentrations in homogenates and Cyt, but those in membranes were spared (Figure 3A). Because of the lower protein yield per mg of tissue in TMs, when ApN was expressed as ng/mg of tissue, the contribution of TMs to the total amount of ApN in whole control tissue (homogenate) was lowered, and yet the relative decrease in ApN levels caused by cAMP remained less pronounced in the TM than in the Cyt fractions (64% and 96% respectively, $P < 0.001$) (Figure 3B).

Table 2 compares the effects of cAMP on ApN secretion and ApN changes in TM and Cyt from explants cultured for 10 h. Under control (basal) conditions, only the 32 kDa species was secreted, while changes in ApN concentrations occurred in TMs. The decrease of ApN in TMs amounted to 1.2 ng/mg of tissue, whereas virtually no change could be observed in Cyt. The ratio of secreted ApN (32 kDa) to depleted ApN in TMs was approx. 4:1 (i.e. ApN secretion largely exceeded ApN depletion of the membrane compartment), suggesting ongoing synthesis of the protein. After 10 h cAMP exposure, secretion of the 32 kDa species was lower than under control conditions (2.3 ng/mg of tissue compared with 5.1 ng/mg) and depletion of ApN in TMs was more pronounced (1.8 ng/mg compared with 1.2 ng/mg). The ratio of secreted ApN (32 kDa) to depleted ApN in TMs was approx. 1:1, which indicates that the whole amount of ApN initially present in membranes was secreted. The Cyt pool was almost completely emptied by cAMP and the immature form of

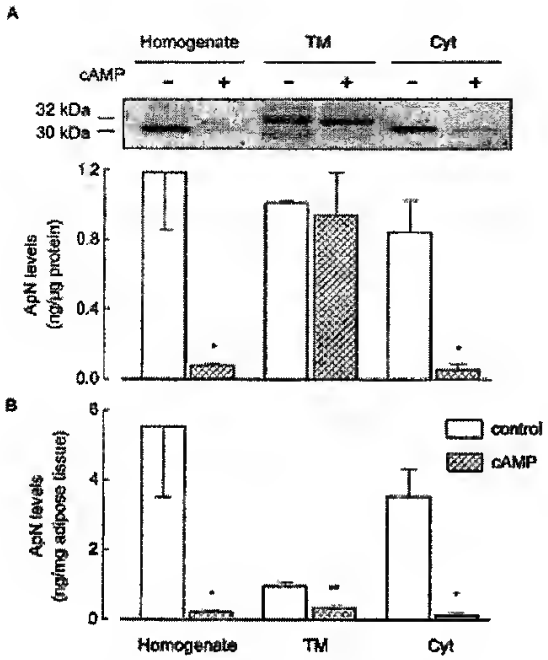


Figure 3 Effect of cAMP on ApN levels in homogenate, TMe and Cyt obtained from explants

Visceral adipose tissue was cultured for 10 h in MEM with or without 1 mM cAMP, then fractionated into TM and Cyt fractions. Homogenate, TM or Cyt proteins (2.5 μg) were loaded on each lane, and autoradiographic signals from Western blots, like that shown in the inset, were quantified by scanning densitometry using rApN as a standard. ApN levels were expressed as ng per mg of protein (A) or in ng/mg of tissue (B). Results are the means ± S.E.M. for three pools of adipose tissue, each composed of four or five mice. °$P = 0.05$, *$P < 0.05$, **$P < 0.01$ for the effect of cAMP.

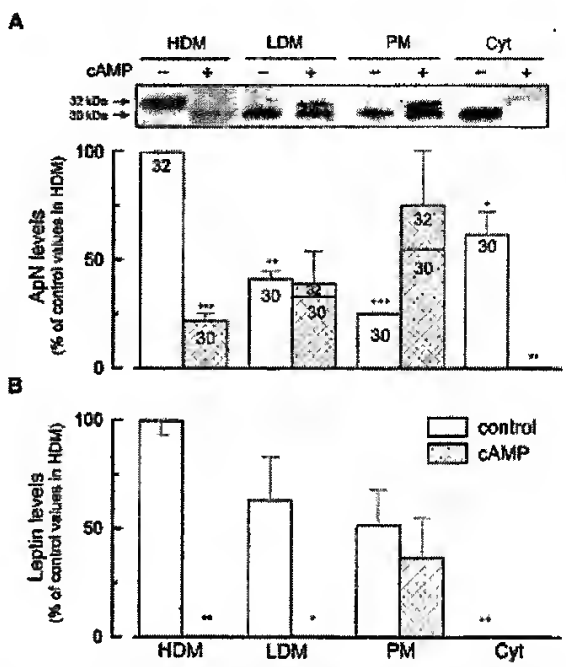


Figure 4 Effect of cAMP on ApN (A) and leptin (B) levels in different subcellular fractions prepared from isolated adipocytes

Visceral explants were cultured for 10 h in MEM without (−, control) or with (+) 1 mM cAMP. At the end of the culture, adipocytes were isolated, and then fractionated by differential ultracentrifugation into HDMs, LDMs, PMs and Cyt. Aliquots of these fractions (5 μg of protein for ApN, 100 μl for leptin) were used for adipocytokine determination by Western blotting (ApN, a representative blot is shown in the inset) or RIA (leptin). Data were calculated as absorbance units (ApN) or ng/ml (leptin) normalized to aliquot protein content. ApN or leptin levels were then expressed as percentages of control values in HDM fractions. The 30 or 32 kDa species of ApN are indicated. Absolute values of leptin concentrations in control HDMs were 2.60 ± 0.19 ng/mg protein. Results are the means ± S.E.M. for three (two for PMs) independent experiments. †$P < 0.05$, ††$P < 0.01$, †††$P < 0.001$ versus control HDM; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ for the effect of cAMP in a given subcellular fraction.

30 kDa was subsequently exported. The ratio of secreted ApN (30 kDa) to depleted ApN in Cyt was approx. 3:5, which suggests intracellular or intra-medium degradation of this species.

In some experiments, we detailed further the effects of cAMP on ApN distribution within the different subcellular membrane compartments (HDMs, LDMs and PMs) in adipocytes isolated from 10 h-cultured explants (Figure 4). Leptin was measured simultaneously for comparison. ApN levels in each adipocyte fraction were calculated as absorbance units/μg of protein, and then expressed relative to control values in HDMs. After 10 h of culture in control medium, ApN was most abundant in HDMs, where it was detected as the 32 kDa species. The 30 kDa species was mainly recovered in the Cyt fraction. On the whole, these

Table 2 Comparison of cAMP effects on ApN secretion and ApN changes in subcellular fractions (SCF) from adipose tissue

Visceral adipose explants were cultured for 10 h in MEM without (control) or with 1 mM cAMP, then fractionated into TM or Cyt fractions. Concentrations of the indicated ApN species secreted in medium by explants over 10 h were expressed as ng/mg of adipose tissue. Changes of ApN concentrations in each subcellular fraction (TM or Cyt) over the culture course were calculated as the initial concentrations (i.e. at 0 h) minus those measured after 10 h and expressed as ng/mg of adipose tissue. Changes of ApN in TMs correspond to those of the 32 kDa/species, and changes in Cyt to those of the 30 kDa species. The initial concentrations of ApN in TMs (32 kDa) and in Cyt (30 kDa) were 2.1 ± 0.1 and 2.5 ± 0.2 ng/mg of adipose tissue respectively. Ratios of secreted ApN (32 or 30 kDa)/depleted ApN in the related subcellular fraction are also presented. Results are the means ± S.E.M. for four pools of adipose tissue, each composed of ten mice. *$P < 0.05$, †$P < 0.01$, ‡$P < 0.001$ for the effect of cAMP.

	Secretion of the indicated ApN species (ng/mg)		Depletion of ApN in each SCF (ng/mg)		Secretion of the indicated ApN species/depletion of ApN in the related SCF	
Conditions	32 kDa	30 kDa	TM	Cyt	32 kDa/TM	30 kDa/Cyt
Control	5.1 ± 0.5	0	1.2 ± 0.3	0	4.2 ± 1.2	–
cAMP	2.3 ± 0.2‡	1.4 ± 0.2‡	1.8 ± 0.2*	2.4 ± 0.9†	1.3 ± 0.1*	0.6 ± 0.1‡

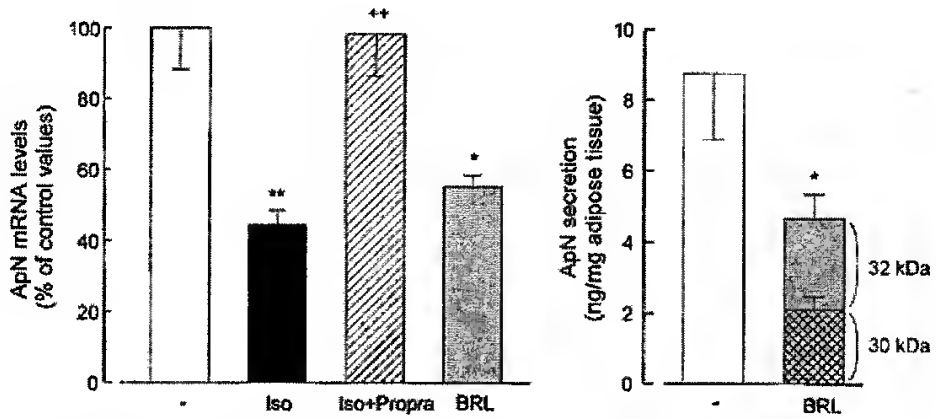


Figure 5 Effects of isoproterenol, propranolol or BRL37344 on ApN gene expression and secretion

Visceral adipose tissue was cultured in MEM without (—, control medium) or with the indicated agents. Concentrations of 10 μM isoproterenol (Iso), 10 μM isoproterenol + 100 μM propranolol (Iso + Propra) or 10 μM BRL37344 (BRL) were added to the medium for 10 h. mRNA levels were expressed as percentages of control values. ApN released into the medium after BRL37344 exposure for 10 h was expressed as ng/mg of adipose tissue. Results are the means ± S.E.M. for three to six pools of adipose tissue, each composed of four or five mice. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control medium; $^{++}P < 0.001$ versus Iso.

Table 3 Inhibition of ApN gene expression by various β-adrenergic agonists in visceral adipose tissue

Explants were cultured for 10 h in MEM without (basal) or with increasing concentrations of various β-adrenergic agonists. The inhibitory effects of dobutamine (β_1), fenoterol (β_2) or BRL37344 (β_3) on ApN mRNA levels were measured. The maximal inhibitory response (I_{max}) corresponds to the % decrease of ApN mRNA from basal values, at the maximal effective concentration of the agonist. The concentration of β-adrenergic agonists inducing 50% of maximal inhibition of ApN mRNA (IC_{50}) is expressed as $-\log_{10} IC_{50}$ in M. Results are the means ± S.E.M. for the numbers of pools of visceral adipose tissue shown in parentheses. Each pool was composed of 20 mice. $^{*}P < 0.01$, significant response with the indicated agent versus basal conditions; $\dagger P < 0.05$, significant differences for the IC_{50} of BRL37344 versus dobutamine.

β-Adrenergic agonists	Maximal inhibitory response I_{max} (% decrease from basal values)	IC_{50} ($-\log_{10} IC_{50}$ in M)
Dobutamine	60.1 ± 9.8* (5)	5.07 ± 0.41 (5)
Fenoterol	18.1 ± 12.6 (5)	5.95 ± 0.36 (5)
BRL37344	67.0 ± 7.6* (5)	6.99 ± 0.40† (5)

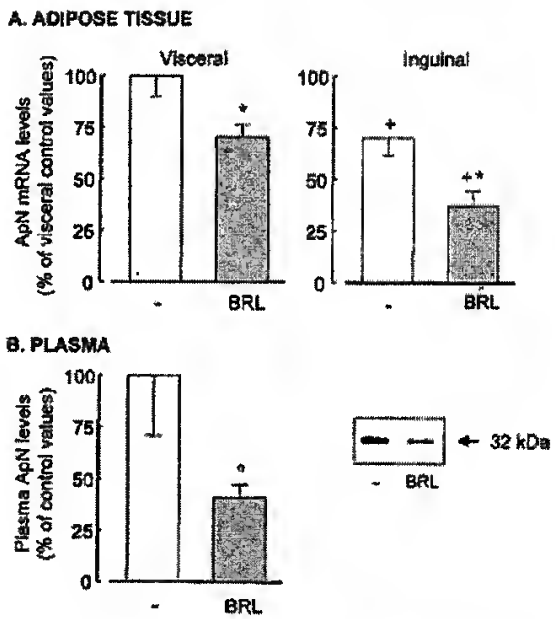


Figure 6 Effect of BRL37344 treatment on adipose tissue ApN gene expression (A) and plasma ApN level (B)

Mice were injected s.c. with BRL37344 (BRL) dissolved in saline (2 mg/kg of body weight, twice at an 8 h interval), while control mice (—) received the vehicle only. Adipose tissue and plasma were collected at the end of the experiment (i.e. 16 h after the first injection). mRNA and plasma levels of ApN were expressed as percentages of values in control mice. The inset shows Western blot of plasma ApN after treatment with BRL or vehicle. Results are the means ± S.E.M. for five control and five BRL mice. $^{*}P = 0.05$, $^{*}P < 0.05$ for the effect of BRL37344 $^{+}P < 0.001$ versus visceral adipose tissue of respective mice.

findings are consistent with those obtained with the other experimental procedure performed on explants as depicted in Figure 3(A). Importantly, leptin was virtually absent from Cyt and only present in intra- and peri-cellular membranes (Figure 4). Relatively high amounts of leptin were found in PMs, at variance with another study [22], because adipocytes were isolated from 10 h-cultured, not fresh, tissue (M. L. Delporte, unpublished work). cAMP induced quantitative and qualitative changes of ApN within adipocytes. cAMP depleted ApN levels in HDM and Cyt fractions (owing to the small number of experiments, the quantitative modifications in PMs were not statistically significant). cAMP also caused changes in the distribution of ApN species into specific subcellular compartments. Thus after 10 h cAMP exposure, only the 30 kDa species was recovered in HDMs, whereas small amounts of the 32 kDa species were found, in addition to the expected 30 kDa form, in LDMs and PMs. cAMP emptied leptin pools in both microsomal fractions.

The inhibitory effects of cAMP on ApN mRNA and secretion were mimicked by β-adrenergic agonists (isoproterenol, a non-specific agonist, or BRL37344, a selective β_3-agent, both used at 10 μM concentrations) and prevented by the addition of propranolol, a β-adrenoceptor antagonist. In the presence of

BRL37344, ApN was secreted in medium as two species of 30 and 32 kDa, in ratios (approx. 3:7) similar to those observed with cAMP (compare Figure 5 and Figure 2E).

The inhibitory effects of several selective β-adrenergic agonists on ApN gene expression were compared after 10 h of culture (Table 3). While fenoterol (a β_2-agonist) had no significant effect, dobutamine (a β_1-agonist) and BRL37344 produced similar maximal inhibitory responses. However, BRL37344 caused a 50% inhibition of ApN mRNA levels at a concentration (approx. 10^{-7} M) two orders of magnitude lower than that required to achieve a similar effect with dobutamine (approx. 10^{-5} M).

The effects of BRL37344 were tested in mice *in vivo* (Figure 6). Mice were injected s.c. twice with BRL37344, and adipose tissue ApN mRNA and plasma ApN levels were measured after 16 h of treatment. Administration of BRL37344 to mice caused a 30–50% reduction of ApN mRNA levels in visceral and s.c. adipose depots and a 60% decrease of ApN levels in plasma (where only the 32 kDa species was detected).

DISCUSSION

cAMP and β-agonists exert a dual (pre- and post-translational) negative effect on ApN secretion in cultured mouse adipose explants. The inhibitory effect was reproduced in mice *in vivo* and in humans *in vitro*.

In the basal state, mouse ApN mRNA levels decreased spontaneously in both adipose tissue (visceral and subcutaneous) sites, whereas 18 S and cyclophilin parameters did not change, arguing against a non-specific decrease of the mRNAs. Although TNFα may potentially be released by explants and inhibit ApN gene expression [27,28], the decrease of ApN mRNA was unaffected by immunoneutralization of the medium with anti-TNFα antibody or inhibition of TNFα production. It was also unaffected when BSA was replaced by FCS in the culture medium (results not shown), ruling out some serum-starved mechanisms. Moreover, it could not reflect glucose deprivation, as glucose concentrations in the medium remained stable, or any other nutritional deficiency, because of the short culture time. This decrease was, however, prevented by actinomycin D, and thus could require ongoing transcription and ensuing synthesis of an inhibitory protein that should act only by destabilizing the mRNA. Similar conclusions have been reached in humans both *in vivo* and *in vitro*, suggesting that this factor may be part of a negative feedback loop by which fat mass itself controls its own ApN production [18,29].

cAMP accelerated the spontaneous decline of the mRNAs. Its effect was unrelated to enhanced lipolysis, but was substantially reversed by actinomycin. cAMP could destabilize the messengers or inhibit the transcription of the ApN gene via direct or indirect mechanisms. If cAMP was only directly destabilizing ApN mRNAs, one would expect no change in the 'blunted' levels of the mRNA when cAMP and actinomycin D were combined. As this was not the case, other mechanisms must be involved. cAMP could directly inhibit ApN gene transcription. No cAMP-response element ('CRE') has been identified in the promoter of the human or mouse ApN gene, but this does not rule out the possibility that the transcription factors activated by cAMP may bind to non-conventional sites [30,31]. Eventually cAMP could act indirectly, possibly through enhanced transcription of the inhibitory protein mentioned above.

In the basal state, a 32 kDa form of ApN was secreted progressively into the medium and subsequently exported in blood. Its sustained secretion may involve ongoing synthesis, as described for leptin [32, 33], and sorting from a vesicular, mainly HDM compartment, a subcellular fraction that contains mostly endoplasmic reticulum [23]. Small amounts of ApN were also associated with downstream membrane localizations (i.e. LDMs and PMs). Our results are thus consistent with those of Bogan et al. [34], who showed, by deconvolution immunofluorescence microscopy in 3T3-L1 cells that ApN partially co-localized with a resident protein of the endoplasmic reticulum while some ApN staining was present in peripheral storage vesicles. Trafficking into the adipocytes appears to be complex; in particular, secretion of some adipocytokines (leptin, adipsin and ApN) may involve both constitutive and regulated exocytosis (i.e. non-canonical pathways) [22,34]. In our experiments, a substantial amount of ApN, in its 30 kDa form, was consistently recovered with the Cyt fraction, although we took the utmost care to avoid vesicular linkage. Importantly, under the same conditions, leptin was confined mainly to microsomes ([24] and the present study) and was virtually absent from this Cyt fraction, which may thus comprise a novel adipocytokine storage pool. This 30 kDa species of ApN was likely to represent an immature form of the protein that did not become modified post-translationally. First, this immature form, unlike the 32 kDa one, was not fated to be exported (i.e. not found in medium or blood, at least under 'normal' or basal conditions). Secondly, its relative molecular mass (M_r) is similar to that of rApN, which rules out the possibility that it may represent a breakdown product of the native protein [3]. Thirdly, during metabolic pulse–chase experiments, a small but reproducible increase in the M_r of ApN has been observed after 20 min of chase, as shown by SDS/PAGE [34]. This is likely to represent hydroxylation of collagen-domain lysine and proline residues, and glycosylation [2,13,35], by analogy to similar modifications in the structurally related mannan-binding protein, which increases from M_r 24000 to 26000 during maturation [36].

cAMP induced both quantitative and qualitative changes in ApN secretion. It decreased the total amount of ApN secreted by inducing a marked depletion of tissue ApN protein and mRNA levels. It also promoted sorting of the immature 30 kDa form by emptying both the Cyt pool, which otherwise remained unaltered, and the HDM fraction that normally did not contain this species. The latter abnormality may be due to cAMP-induced redistribution of ApN species into the different vesicular compartments (30 kDa form targeted to HDMs, small aberrant 32 kDa amounts found in LDMs and PMs). This 'mistargeting' may result from impairment of protein maturation that otherwise provides the code for ultimate protein destination [37]. Whether other agents or hormones that negatively regulate ApN also promote sorting of an immature form of the protein is still unresolved. A single band of ApN has been detected by Western blot analysis of medium samples from 3T3-L1 adipocytes treated with TNFα or dexamethasone [28]. However, this does not rule out the possibility that the two ApN species could be secreted, since extended running times for gel electrophoresis are required to detect small differences in M_r. Moreover, differences in culture models (3T3-L1 compared with mature adipose tissue) or in the antibody used may also contribute.

Figure 5 shows that, like cAMP, β-adrenoceptor activation inhibited ApN gene expression in cultured mouse explants, in agreement with recent results obtained in 3T3-L1 cells [12]. ApN gene down-regulation was dependent on β_1- and β_3-adrenoceptors, and was mainly driven by the latter. These findings are consistent with functional studies on β-adrenoceptor subtype-mediated lipolysis or inhibition of *ob* gene expression in white and brown adipocytes of rodents [38–40]. Like cAMP, β_3-adrenergic stimulation also inhibited ApN secretion and split protein release into two species of different molecular masses.

On the whole, the effects *in vitro* of BRL37344 were reproduced *in vivo*. Thus administration s.c. of BRL37344 to mice caused a 30–50 % decrease in ApN mRNA levels in both adipose tissue sites, and a concomitant decrease in plasma ApN levels. However, only the mature 32 kDa form of ApN, and not the 30 kDa one, was detected in blood. One may speculate that the immature 30 kDa species was less stable and thus more prone to degradation, a hypothesis consistent with our results *in vitro*. This is reminiscent of the reduced stability of non-hydroxylated collagen resulting from impaired peptidyl hydroxylation attributed to vitamin C deficiency and responsible for many of the clinical findings of scurvy [41]. It is noteworthy that calculated concentrations of circulating BRL37344 (on the basis of mean mouse circulating volume and mean s.c resorption) averaged approx. 10^{-5} M, a concentration close to that found to be efficient *in vitro*.

Lastly, we extended some of our results to humans. cAMP exerted a marked inhibition on ApN mRNA in human visceral adipose explants, in agreement with the decreased content and secretion of the adipocytokine reported in human preadipocytes [42]. We evaluated further the effects of β-adrenoceptor stimulation, which have not been performed in human fat. Owing to limited tissue availability, only isoproterenol was tested. ApN mRNA was negatively regulated by the β-agonist, which supports an inhibitory effects of catecholamines on the human gene.

Epidemiological studies strongly suggest that stress influences the development and progression of carotid and coronary atherosclerosis [43,44]. Stress and catecholamines could therefore contribute to decrease plasma ApN levels. In this case, hypoadiponectinaemia would be a novel link between stress and atherosclerosis. On the other hand, reducing adiponectinaemia may also be a novel mechanism by which catecholamines affect fuel homoeostasis and insulin sensitivity. Both β-adrenergic agonists and ApN increase thermogenesis and lipid oxidation [3,5,45]. In addition to β-adrenoceptor desensitization, catecholamines' inhibition of ApN production and maturation may be another negative feedback loop to limit fuel and energy spillover. Catecholamines also induce insulin resistance. Their ability to inhibit an insulin-sensitizing adipocytokine, ApN, may impair insulin signalling further [12], thereby potentially contributing to the pathogenesis of insulin resistance and the insulin resistance syndrome.

In conclusion, β-adrenergic stimulation inhibited ApN production and maturation. This may have an important role in stress-induced atherogenesis, fuel homoeostasis and the insulin resistance syndrome.

We are grateful to Professor M. F. van den Hove and Professor M. Wibo for helpful discussion and suggestions on fractionation experiments; to Dr C. M. Halleux for preliminary experiments; and to A. M. Pottier for technical assistance. This work was supported by grants from the Foundation of Scientific and Medical Research (3.4592.99), from the Fonds National de la Recherche Scientifique (1.5.143.00 and 1.5.176.01), and the Fund for Scientific Development (University of Louvain). M.-L.D. is Boursier from the FRIA.

REFERENCES

1 Trayhurn, P. and Beattie, J. H. (2001) Physiological role of adipose tissue: white adipose tissue as an endocrine and secretory organ. Proc. Nutr. Soc. **60**, 329–339
2 Berg, A. H., Combs, T. P. and Scherer, P. E. (2002) ACRP30/adiponectin: an adipokine regulating glucose and lipid metabolism. Trends Endocrinol. Metab. **13**, 84–89
3 Fruebis, J., Tsao, T. S., Javorschi, S., Ebbets-Reed, D., Erickson, M. R., Yen, F. T., Bihain, B. E. and Lodish, H. F. (2001) Proteolytic cleavage product of 30-kDa adipocyte complement-related protein increases fatty acid oxidation in muscle and causes weight loss in mice. Proc. Natl. Acad. Sci. U.S.A. **98**, 2005–2010
4 Berg, A. H., Combs, T. P., Du, X., Brownlee, M. and Scherer, P. E. (2001) The adipocyte-secreted protein Acrp30 enhances hepatic insulin action. Nat. Med. **7**, 947–953
5 Yamauchi, T., Kamon, J., Waki, H., Terauchi, Y., Kubota, N., Hara, K., Mori, Y., Ide, T., Murakami, K. and Tsuboyama-Kasaoka, N. (2001) The fat-derived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity. Nat. Med. **7**, 941–946
6 Ouchi, N., Kihara, S., Arita, Y., Maeda, K., Kuriyama, H., Okamoto, Y., Hotta, K., Nishida, M., Takahashi, M. and Nakamura, T. (1999) Novel modulator for endothelial adhesion molecules: adipocyte-derived plasma protein adiponectin. Circulation **100**, 2473–2476
7 Ouchi, N., Kihara, S., Arita, Y., Okamoto, Y., Maeda, K., Kuriyama, H., Hotta, K., Nishida, M., Takahashi, M., Muraguchi, M. et al. (2000) Adiponectin, an adipocyte-derived plasma protein inhibits endothelial NF-kappa B signalling through a cAMP-dependent pathway. Circulation **102**, 1296–1301
8 Arita, Y., Kihara, S., Ouchi, N., Takahashi, M., Maeda, K., Miyagawa, J., Hotta, K., Shimomura, I., Nakamura, T. and Miyaoka, K. (1999) Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity. Biochem. Biophys. Res. Commun. **257**, 79–83
9 Hotta, K., Funahashi, T., Arita, Y., Takahashi, M., Matsuda, M., Okamoto, Y., Iwahashi, H., Kuriyama, H., Ouchi, N. and Maeda, K. (2000) Plasma concentrations of a novel, adipose-specific protein, adiponectin, in type 2 diabetic patients. Arterioscler. Thromb. Vasc. Biol. **20**, 1595–1599
10 Kissebah, A. H., Sonnenberg, G. E., Myklebust, J., Goldstein, M., Broman, K., James, R. G., Marks, J. A., Krakower, G. R., Jacob, H. J. and Weber, J. (2000) Quantitative trait loci on chromosomes 3 and 17 influence phenotypes of the metabolic syndrome. Proc. Natl. Acad. Sci. U.S.A. **97**, 14478–14483
11 Vionnet, N., Hani, E., Dupont, S., Gallina, S., Francke, S., Dotte, S., De Matos, F., Durand, E., Lepretre, F. and Lecoeur, C. (2000) Genome-wide search for type 2 diabetes-susceptibility genes in French whites: evidence for a novel susceptibility locus for early-onset diabetes on chromosome 3q27-qter and independent replication of a type 2-diabetes locus on chromosome 1q21-q24. Am. J. Hum. Genet. **67**, 1470–1480
12 Fasshauer, M., Klein, J., Neumann, S., Eszlinger, M. and Paschke, R. (2001) Adiponectin gene expression is inhibited by beta-adrenergic stimulation via protein kinase A in 3T3-L1 adipocytes. FEBS Lett. **507**, 142–146
13 Wang, Y., Xu, A., Knight, C., Xu, L. Y. and Cooper, G. J. (2002) Hydroxylation and glycosylation of the four conserved lysine residues in the collagenous domain of adiponectin. Potential role in the modulation of its insulin-sensitizing activity. J. Biol. Chem. **277**, 19521–19529
14 Mandrup, S., Loftus, T. M., MacDougald, O. A., Kuhajda, F. P. and Lane, M. D. (1997) Obese gene expression at *in vivo* levels by fat pads derived from s.c. implanted 3T3-F442A preadipocytes. Proc. Natl. Acad. Sci. U.S.A. **94**, 4300–4305
15 Foufelle, F., Gouhot, B., Pegorier, J. P., Perdereau, D., Girard, J. and Ferré, P. (1992) Glucose stimulation of lipogenic enzyme gene expression in cultured white adipose tissue. A role for glucose 6-phosphate. J. Biol. Chem. **267**, 20543–20546
16 Halleux, C. M., Servais, L., Reul, B. A., Detry, R. and Brichard, S. M. (1998) Multihormonal control of *ob* gene expression and leptin secretion from cultured human visceral adipose tissue: increased responsiveness to glucocorticoids in obesity. J. Clin. Endocrinol. Metab. **83**, 902–910
17 Reul, B. A., Ongemba, L. N., Pottier, A. M., Henquin, J. C. and Brichard, S. M. (1997) Insulin and insulin-like growth factor 1 antagonize the stimulation of *ob* gene expression by dexamethasone in cultured rat adipose tissue. Biochem. J. **324**, 605–610
18 Halleux, C. M., Takahashi, M., Delporte, M. L., Detry, R., Funahashi, T., Matsuzawa, Y. and Brichard, S. M. (2001) Secretion of adiponectin and regulation of apM1 gene expression in human visceral adipose tissue. Biochem. Biophys. Res. Commun. **288**, 1102–1107
19 Jonas, J. C., Sharma, A., Hasenkamp, W., Ilkova, H., Patane, G., Laybutt, R., Bonner-Weir, S. and Weir, G. C. (1999) Chronic hyperglycemia triggers loss of pancreatic beta cell differentiation in an animal model of diabetes. J. Biol. Chem. **274**, 14112–14121
20 Le Marchand-Brustel, Y., Dlichon-Berthe, C., Gremeaux, T., Tanti, J. F., Rochet, N. and Van Obberghen, E. (1990) Glucose transporter in insulin sensitive tissues of lean and obese mice. Effect of the thermogenic agent BRL 26830A. Endocrinology **127**, 2687–2695
21 Halleux, C. M., Declerck, P. J., Tran, S. L., Detry, R. and Brichard, S. M. (1999) Hormonal control of plasminogen activator inhibitor-1 gene expression and production in human adipose tissue: stimulation by glucocorticoids and inhibition by catecholamines. J. Clin. Endocrinol. Metab. **84**, 4097–4105
22 Roh, C., Roduit, R., Thorens, B., Fried, S. and Kandror, K. V. (2001) Lipoprotein lipase and leptin are accumulated in different secretory compartments in rat adipocytes. J. Biol. Chem. **276**, 35990–35994
23 Simpson, I. A., Yver, O. R., Hissin, P. J., Wardzala, L. J., Karnieli, E., Salans, L. B. and Cushman, S. W. (1983) Insulin-stimulated translocation of glucose transporters in the isolated rat adipose cells: characterization of subcellular fractions. Biochim. Biophys. Acta **763**, 393–407

24 Roh, C., Thoidis, G., Farmer, S. R. and Kandror, K. V. (2000) Identification and characterization of leptin-containing intracellular compartment in rat adipose cells Am. J. Physiol. Endocrinol. Metab. **279**, E693–E699
25 Maeda, K., Okubo, K., Shimomura, I., Funahashi, T., Matsuzawa, Y. and Matsubara, K. (1996) cDNA cloning and expression of a novel adipose specific collagen-like factor, apM1 (AdiPose Most abundant Gene transcript 1). Biochem. Biophys. Res. Commun. **221**, 286–289
26 Cigolini, M., Tonoli, M., Borgato, L., Frigotto, L., Manzato, F., Zeminian, S., Cardinale, C., Camin, M., Chiaramonte, E. and De Sandre, G. (1999) Expression of plasminogen activator inhibitor-1 in human adipose tissue: a role for TNF-alpha? Atherosclerosis **143**, 81–90
27 Fasshauer, M., Klein, J., Neumann, S., Eszlinger, M. and Paschke, R. (2002) Hormonal regulation of adiponectin gene expression in 3T3-L1 adipocytes Biochem. Biophys. Res. Commun. **290**, 1084–1089
28 Maeda, N., Takahashi, M., Funahashi, T., Kihara, S., Nishizawa, H., Kishida, K., Nagaretani, H., Matsuda, M., Komuro, R. and Ouchi, N. (2001) PPARgamma ligands increase expression and plasma concentrations of adiponectin, an adipose-derived protein. Diabetes **50**, 2094–2099
29 Yang, W. S., Lee, W. J., Funahashi, T., Tanaka, S., Matsuzawa, Y., Chao, C. L., Chen, C. L., Tai, T. Y. and Chuang, L. M. (2001) Weight reduction increases plasma levels of an adipose-derived anti-inflammatory protein, adiponectin. J. Clin. Endocrinol. Metab. **86**, 3815–3819
30 Das, K., Lin, Y., Widen, E., Zhang, Y. and Scherer, P. E. (2001) Chromosomal localization, expression pattern, and promoter analysis of the mouse gene encoding adipocyte-specific secretory protein Acrp30. Biochem. Biophys. Res. Commun. **280**, 1120–1129
31 Scheffler, A., Langmann, T., Palitzsch, K. D., Scholmerich, J. and Schmitz, G. (1998) Identification and characterization of the human adipocyte apM-1 promoter Biochim Biophys. Acta **1399**, 187–197
32 Russell, C. D., Ricci, M. R., Brolin, R. E., Magill, E. and Fried, S. K. (2001) Regulation of the leptin content of obese human adipose tissue. Am. J. Physiol Endocrinol. Metab. **280**, E399–E404
33 Turban, S., Hainault, I., André, J., Ferré, P., Quignard-Boulangé, A. and Guerre-Millo, M. (2001) Molecular and cellular mechanisms of adipose secretion: comparison of leptin and angiotensinogen. J. Cell. Biochem. **82**, 666–673
34 Bogan, J. S. and Lodish, H. F. (1999) Two compartments for insulin-stimulated exocytosis in 3T3-L1 adipocytes by endogenous ACRP30 and GLUT4. J. Cell Biol. **146**, 609–620
35 Sato, C., Yasukawa, Z., Honda, N., Matsuda, T. and Kitajima, K. (2001) Identification and adipocyte differentiation-dependent expression of the unique disialic acid residue in an adipose tissue-specific glycoprotein, adipo Q. J. Biol. Chem. **276**, 28849–28856
36 Colley, K. J. and Baenziger, J. U. (1987) Identification of the post-translational modifications of the core-specific lectin. The core-specific lectin contains hydroxyproline, hydroxylysine, and glucosylgalactosylhydroxylysine residues. J. Biol. Chem. **262**, 10290–10295
37 Ganong, W. F. (1993) The general and cellular basis of medical physiology. In Review of Medical Physiology, pp. 1–41, Prentice Hall International (UK) Limited, London
38 Deng, C., Moinat, M., Curtis, L., Nadakal, A., Preitner, F., Boss, O., Assimacopoulos-Jeannet, F., Seydoux, J. and Giacobino, J. P. (1997) Effects of beta-adranoceptor subtype stimulation on obese gene messenger ribonucleic acid and on leptin secretion in mouse brown adipocytes differentiated in culture. Endocrinology **138**, 548–552
39 Germack, R., Starzec, A. B., Vassy, R. and Perret, G. Y. (1997) Beta-adrenoceptor subtype expression and function in rat white adipocytes. Br. J. Pharmacol. **120**, 210–210
40 Lafontan, M. and Berlan, M. (1993) Fat cell adrenergic receptors and the control of white and brown fat cell function. J. Lipid Res. **34**, 1057–1091
41 Wilson, J. D. (1983) Disorders of vitamins: deficiency, excess, and errors of metabolism. In Harrison's Principles of Internal Medicine (Petersdorf, R. G., Adams, R. D., Braunwald, E., Isselbacher, K. J., Martin J. B. and Wilson, J. D., eds.), pp. 461–470, McGraw-Hill International Book Company, London
42 Kappes, A. and Lottler, G. (2000) Influences of ionomycin, dibutyryl-cycloAMP and tumour necrosis factor-alpha on intracellular amount and secretion of apM1 in differentiating primary human preadipocytes. Horm. Metab. Res. **32**, 548–554
43 Calvert, G. M., Merling, J. W. and Burnett, C. A. (1999) Ischemic heart disease mortality and occupation among 16- to 60-year-old males. J. Occup. Environ. Med. **41**, 960–966
44 Castillo-Richmond, A., Schneider, R. H., Alexander, C. N., Cook, R., Myers, H., Nidich, S., Haney, C., Rainforth, M. and Salerno, J. (2000) Effects of stress reduction on carotid atherosclerosis in hypertensive African Americans. Stroke **31**, 568–573
45 Schiffelers, S. L., Saris, W. H., Boomsma, F. and van Baak, M. A. (2001) Beta1- and beta2-adrenoceptor-mediated thermogenesis and lipid utilization in obese and lean men. J. Clin. Endocrinol. Metab. **86**, 2191–2199

Received 17 April 2002/16 July 2002; accepted 24 July 2002
Published as BJ Immediate Publication 24 July 2002, DOI 10.1042/BJ20020610

THE JOURNAL OF BIOLOGICAL CHEMISTRY
© 2002 by The American Society for Biochemistry and Molecular Biology, Inc.

Vol. 277, No. 40, Issue of October 4, pp. 37487–37491, 2002
Printed in U.S.A.

Role of Adiponectin in Preventing Vascular Stenosis

THE MISSING LINK OF ADIPO-VASCULAR AXIS*

Received for publication, June 19, 2002
Published, JBC Papers in Press, July 22, 2002, DOI 10.1074/jbc.M206083200

Morihiro Matsuda‡§, Iichiro Shimomura‡§¶, Masataka Sata‖, Yukio Arita‡, Makoto Nishida‡, Norikazu Maeda‡, Masahiro Kumada‡, Yoshihisa Okamoto‡, Hiroyuki Nagaretani‡, Hitoshi Nishizawa‡, Ken Kishida‡, Ryutaro Komuro‡, Noriyuki Ouchi‡, Shinji Kihara‡, Ryozo Nagai‖, Tohru Funahashi‡, and Yuji Matsuzawa‡

From the ‡Department of Internal Medicine and Molecular Science, Graduate School of Medicine, Osaka University, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan and the ‖Department of Cardiovascular Medicine, University of Tokyo Graduate School of Medicine, 7-3-1 Hongo, Bunkyoku, Tokyo 113-8655, Japan

Obesity is more linked to vascular disease, including atherosclerosis and restenotic change, after balloon angioplasty. The precise mechanism linking obesity and vascular disease is still unclear. Previously we have demonstrated that the plasma levels of adiponectin, an adipose-derived hormone, decreases in obese subjects, and that hypoadiponectinemia is associated to ischamic heart disease. In current the study, we investigated the *in vivo* role of adiponectin on the neointimal thickening after artery injury using adiponectin-deficient mice and adiponectin-producing adenovirus. Adiponectin-deficient mice showed severe neointimal thickening and increased proliferation of vascular smooth muscle cells in mechanically injured arteries. Adenovirus-mediated supplement of adiponectin attenuated neointimal proliferation. In cultured smooth muscle cells, adiponectin attenuated DNA synthesis induced by growth factors including platelet-derived growth factor, heparin-binding epidermal growth factor (EGF)-like growth factor (HB-EGF), basic fibroblast growth factor, and EGF and cell proliferation and migration induced by HB-EGF. In cultured endothelial cells, adiponectin attenuated HB-EGF expression stimulated by tumor necrosis factor α. The current study suggests an adipo-vascular axis, a direct link between fat and artery. A therapeutic strategy to increase plasma adiponectin should be useful in preventing vascular restenosis after angioplasty.

Obesity is a common risk for insulin resistance and cardiovascular diseases (1, 2). However, the molecular mechanism of the relationship between obesity and vascular diseases remains unclear. Adipocytes produce and secrete a variety of biologically active molecules, conceptualized as adipocytokines, including tumor necrosis factor (TNF) α,[1] leptin, resistin, plasminogen activator inhibitor-1 and heparin-binding epidermal growth factor (EGF)-like growth factor (HB-EGF) (3–7). Several lines of evidences suggest that dysregulated production of adipocytokines participates in the development of metabolic and vascular diseases related to obesity (3–7).

Adiponectin is an adipocyte-derived factor that was identified by our group in human adipose tissues (8). Acrp30 or AdipoQ, independently cloned by two groups, is the mouse counterpart of adiponectin (9, 10). Adiponectin mRNA is expressed exclusively in adipose tissues. Adiponectin is composed of two structurally distinct domains: C-terminal collagen-like fibrous domain and complement C1q-like globular domain. Interestingly, low plasma concentrations of adiponectin are found in obese subjects (11) and patients with coronary artery disease (12). Furthermore, the incidence of cardiovascular death is higher in renal failure patients with low plasma adiponectin compared with those with higher plasma adiponectin levels (13). We have also reported that adiponectin infiltrates rapidly into the subendothelial space of the vascular wall when the endothelial barrier of the arterial wall is injured by balloon angioplasty (14). In tissue cultures, adiponectin attenuates monocyte attachment to endothelial cells by reducing the expression of adhesion molecules on endothelial cells (12, 15). Adiponectin also suppresses lipid accumulation in monocyte-derived macrophages through the suppression of macrophage scavenger receptor expression (16). These *in vitro* data suggested the anti-atherogenic properties of adiponectin, and hence hypoadiponectinemia might be associated with a higher incidence of vascular diseases in obese subjects.

In the present study, we investigated the role of adiponectin on the vascular wall *in vivo* using adiponectin knockout (KO) mice and adiponectin-producing adenovirus (17). Our results demonstrate that adiponectin deficiency aggravates neointimal thickening, and adiponectin supplement attenuates neointimal thickening in mechanically injured arteries, presumably through the suppressive effect of adiponectin on the proliferation and migration of vascular smooth muscle cells. Here we show the first *in vivo* evidence that adiponectin is a fat-derived hormone directly bridging the adipose-vasculer axis.

* This work was supported in part by "Research for the Future" Program JSPS-RFTF97L00801 from the Japan Society for the Promotion of Science and Grants-in-aid 12307022, 12557090, and 12671084 from the Ministry of Education, Science, Sports and Culture of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Current address: Dept. of Organismal Biosystems, Graduate School of Frontier Bioscience, Dept. of Medicine and Pathophysiology, Graduate School of Medicine, Osaka University, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan.

¶ To whom correspondence should be addressed. Tel.: 81-6-6879-3270 or -3272; Fax: 81-6-6879-3279; E-mail: ichi@fbs.osaka-u.ac.jp.

[1] The abbreviations used are: TNFα, tumor necrosis factor α; EGF, epidermal growth factor; HB-EGF, heparin-binding EGF-like growth factor; KO, knockout; WT, wild-type; APN, adiponectin; Ad-APN, adenovirus-adiponectin; Ad-βgal, adenovirus-β-galactosidase; HASMC, human aortic smooth muscle cell; HAEC, human aortic endothelial cell; PDGF, platelet-derived growth factor; FGF, fibroblast growth factor; I/M ratio, intimal/medial area ratio; VSMC, vascular smooth muscle cell.

TABLE I
Phenotypic comparison in wild-type and adiponectin knockout mice

Wild-type and adiponectin knockout mice were sacrificed at 16 weeks old in the non-fasted (*ad libitum*) and fasted (12 h-fasted) state ($n = 4$). ND, not determined. Each value is mean ± S.E. FFA, free fatty acid.

	Non-fasted		12-h fasted	
	Wild-type	Knockout	Wild-type	Knockout
Number and gender	4 male	4 male	4 male	4 male
Body weight (g)	30.0 ± 2.2	30.9 ± 0.9	26.0 ± 1.5	28.5 ± 1.9
Epididymal fat (g)	0.65 ± 0.07	0.67 ± 0.08	0.50 ± 0.02	0.77 ± 0.17
Brown fat (g)	0.40 ± 0.02	0.37 ± 0.15	0.34 ± 0.02	0.35 ± 0.01
Liver (g)	1.74 ± 0.16	1.74 ± 0.84	1.31 ± 0.08	1.43 ± 0.10
Gastrocnemius muscle (g)	0.59 ± 0.01	0.59 ± 0.06	0.61 ± 0.02	0.60 ± 0.01
Heart (g)	0.13 ± 0.00	0.15 ± 0.01	0.13 ± 0.01	0.14 ± 0.01
Plasma glucose (mg/dl)	202.8 ± 4.6	250.7 ± 18.3	121.4 ± 2.8	107.5 ± 15.8
Plasma insulin (ng/ml)	0.20 ± 0.04	0.32 ± 0.14	0.05 ± 0.03	0.03 ± 0.01
Plasma triglyceride (mg/dl)	37.1 ± 6.5	48.4 ± 7.0	28.1 ± 4.6	28.0 ± 5.8
Plasma cholesterol (mg/dl)	ND	ND	58.1 ± 5.9	60.1 ± 6.5
FFA (mM)	0.92 ± 0.22	0.76 ± 0.18	1.14 ± 0.32	0.95 ± 0.13

EXPERIMENTAL PROCEDURES

Animals—Adiponectin KO male mice (8–11 weeks old) were generated as described previously (17). Briefly, the targeting vector for the adiponectin KO mice was constructed using a positive selection cassette derived from a vector, pPolIIsneobpA, containing the neo^R gene. The 1.4-kb *Vsp*I-*Eco*RI region, located in intron 2 of mouse adiponectin gene, was inserted into the *Vsp*I site of pPolIIsneobpA prior to the neo^R cassette. The 7-kb *Sna*BI-*Aat*II region located in intron 1 was inserted into the *Sna*BI site to the 3′ site of the neo^R cassette. The targeting construct was linearized with *Not*I and introduced into mouse AB2.2-prime embryonic stem cells (Lexicon Genetics, Woodlands, TX) by electroporation (270 V, 500 microfarad, BTX ECM600). Embryonic stem cell clones resistant to G418/gancyclovir were isolated, and 16 positive clones were obtained. Chimeric animals obtained from the microinjections were bred to C57BL/6J mice, and three chimeric males sired offspring that carried the disrupted mouse adiponectin allele through the germ line.

Femoral Artery Injury—The femoral artery injury procedure in mice was conducted as described previously (18, 19). Briefly, wild-type (WT) and adiponectin KO male mice underwent bilateral femoral artery injury by a straight spring wire (0.36-mm diameter, no. SKI 175 FLP 14-S, Invatec, Concesio (BS), Italy), denuding vascular endothelium and inducing neointimal hyperplasia. At 2–3 weeks after vascular injury, the mice were anesthetized, and both femoral arteries were harvested after perfusion fixation with 10% formalin and embedded in paraffin. Following embedding in paraffin, parallel sections were stained with hematoxylin and eosin. Smooth muscle cells were identified by immunostaining for α-smooth muscle actin using clone 1A4 from Sigma as the primary antibody. Intimal and medial area were measured using the image analysis software MacSCOPE.

BrdUrd Staining—Following vascular injury, 100 μg/g BrdUrd was administered intraperitoneally every 24 h until harvesting the femoral arteries. On the 14th day after vascular injury, the femoral arteries were perfusion-fixed in 10% formalin, harvested, and embedded in paraffin. After deparaffinization, parallel sections were immunostained with BrdUrd using a BrdUrd staining kit (Oncogene Research Products, Boston, MA). BrdUrd-labeled and -unlabeled smooth muscle cells in neointima were counted for each section. The proliferation index was calculated by dividing the number of BrdUrd-labeled cells by the number of unlabeled cells as described previously (20).

Preparation and Administration of Adenovirus—Adenovirus producing the full-length mouse adiponectin was prepared by using the Adenovirus Expression Vector kit (Takara, Kyoto, Japan). 2×10^8 plaque-forming units of adenovirus-adiponectin (Ad-APN) or adenovirus-β-galactosidase (Ad-βgal) was injected into the jugular vein of mice 3 days prior to the femoral artery injury. On the 14th day after the virus injection (11th day after the injury), the femoral arteries were harvested for analysis.

Cell Culture—Human aortic smooth muscle cells (HASMCs) (Clonetics) were maintained and used for experiments at passage 4 or 5 as previously described (21). Human aortic endothelial cells (HAECs) (Clonetics) were maintained in plastic plates precoated with type I collagen (BD PharMingen) as described previously (15). Human recombinant adiponectin was prepared as reported previously (11).

Cell Proliferation Assays—HASMCs were treated for 18 h in Dulbecco's modified Eagle's medium containing 2% fetal calf serum (Invitrogen) with or without 10 ng/ml human recombinant platelet-derived growth factor (PDGF)-BB, HB-EGF, basic fibroblast growth factor (FGF), and EGF (R&D Systems) in the presence or absence of 30 μg/ml human recombinant adiponectin. The cells were exposed to [^{3}H]thymidine (Amersham Biosciences) at 20 μCi/ml for 8, then trypsinized, and retrieved onto glass fiber filters using an automatic cell harvester. [^{3}H]Thymidine uptake was measured in a direct β counter. Cell number was counted with the hemocytometer method as described previously (22).

Cell Migration Assay—Migration assays were performed using a Boyden chamber. HASMCs (5×10^4 cells/ml) were added to the Transwell inserts (Costar, 12-mm diameter, 12.0-μm pore size) precoated with collagen type I. Migration was induced by HB-EGF (10 ng/ml) with or without adiponectin (30 μg/ml) added to the lower chamber beneath the insert membrane. The Transwell chambers were then incubated for 4 h under culture condition. Migrated HASMCs on the lower surface of the membrane were fixed with ethanol and stained with hematoxylin. Migration activity was evaluated microscopically by counting the number of stained nuclei per high power field (×400). All assays were performed in triplicate, and each sample was counted randomly in 10 different areas in the center of the membrane.

Measurement of HB-EGF mRNA—HAECs in a confluent state were preincubated for 16 h in medium M199 (Invitrogen) containing 0.5% fetal calf serum and 3% bovine serum albumin with or without 30 μg/ml recombinant adiponectin and then exposed to human recombinant TNFα (R&D Systems) or vehicle at a final concentration of 10 ng/ml for 2 h. Cells were harvested, and total RNA was prepared with an RNA STAT-60 kit (Tel-Test, Friendswood, TX). cDNA was produced using Taqman reverse transcription kits (PerkinElmer Life Sciences). Real-time PCR was performed on an ABI-Prism 7700 using the Master Mix SYBR Green kit (PE-Applied Biosystems, Norwalk, CT) according to the manufacturer's instructions. Primers were: 5′-TGCTGCAAGCCA-CAAGCACT-3′ and 5′-GCCCATGACACCTCTCTCCA-3′ for HB-EGF and 5′-ACCACAGTCCATGCCATCAC-3′ and 5′-CACCACCTTCTT-GATGTCATC-3′ for glyceraldehyde-3-phosphate dehydrogenase.

Statistical Analysis and Ethical Considerations—Results were expressed as mean ± S.E. Differences between groups were examined for statistical significance using the Student's *t* test or analysis of variance with Fisher's protected least significant difference test. A p value less than 0.05 denoted the presence of a statistically significant difference. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Osaka University School of Medicine.

RESULTS

Basal Profile of Adiponectin Knockout Mice—Adipose mRNA and plasma protein of adiponectin were deficient in KO mice studied in the current analysis (data not shown). Table I describes the phenotypic comparison in WT and adiponectin KO mice under non-fasted and 12-h fasted conditions. No significant differences were observed in the weights of body and various tissues including epididymal white fat, brown fat, liver, gastrocnemius muscle, and heart. Plasma concentration of glucose, insulin, cholesterol, triglyceride, and free fatty acid were not altered significantly in the adiponectin KO mice.

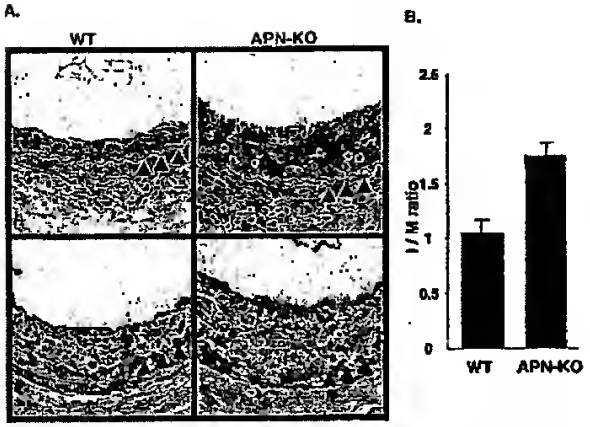


FIG. 1. **Intimal thickening of injured arteries in adiponectin-deficient mice.** *A*, representative hematoxylin and eosin-stained sections (*upper panel*) and α-smooth muscle-actin immunostained sections (*lower panel*) of femoral arteries of WT and adiponectin knockout (*APN-KO*) male mice (8–10 weeks old) harvested at 3 weeks after injury. *Arrows* indicate internal elastic lamina. *B*, I/M ratio in the injured arteries of WT (n = 7, ±S.E.) and APN-KO (n = 5, ±S.E.) mice ($p < 0.01$, t test).

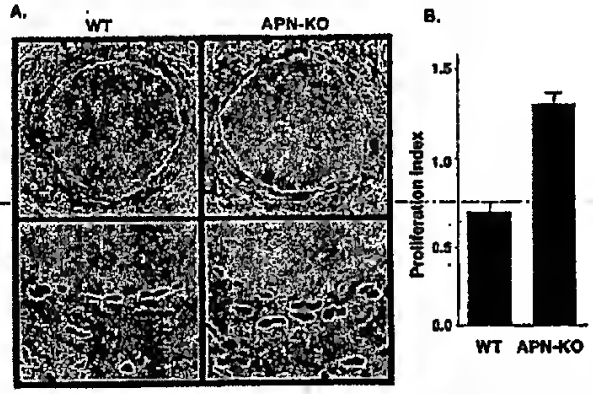


FIG. 2. **Neointimal proliferation of injured arteries in adiponectin-deficient mice.** *A*, representative BrdUrd-stained sections (*upper panel*, ×100; *lower panel*, ×400) of femoral arteries in WT and APN-KO male mice (11–13 weeks old) harvested at 2 weeks after injury counterstained with hematoxylin. *B*, proliferation index (the ratio of the number of BrdUrd-labeled/unlabeled cells) measured from BrdUrd-stained sections of WT (n = 5, ± S.E.) and APN-KO (n = 5, ± S.E.) mice (p = 0.001, t test).

Adiponectin Deficiency Increases Neointimal Thickening in Injured Arteries—In the present study, we denuded the vascular endothelium of the femoral artery as we described previously (19, 20) and compered the neointimal thickening of the arteries at 3 weeks after the injury between WT and KO mice. Hematoxylin-eosin staining (Fig. 1*A*, *upper panel*) demonstrated that the neointimal hyperplasie in the injured artery was worse in KO mice than in WT mice. Immunohistochemical staining revealed thet the neointima in both WT and KO mice wes composed of α-smooth muscle actin-positive smooth muscle cells (Fig. 1*A*, *lower panel*). We also quantitatively measured the intimal snd medisl srea by computerized morphometry (Fig. 1*B*). The I/M ratio (the retio of intimal area/medial area) wes significantly greater in KO mice compared with WT mice ($p < 0.01$). Notably, two of seven injured femoral arteries were occluded only in KO mice at 3 weeks efter injury.

Adiponectin Deficiency Increases Proliferation of Vascular Smooth Muscle Cells in Injured Arteries—Next we essessed

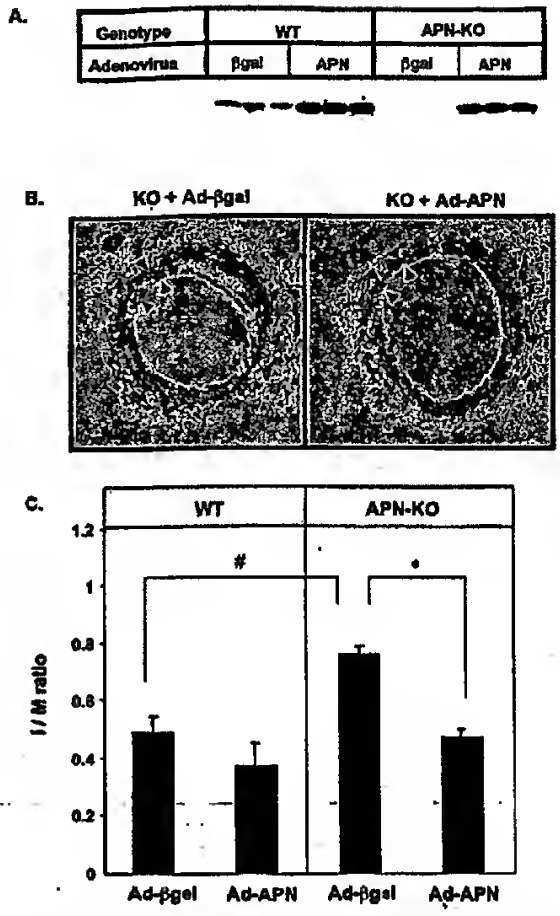


FIG. 3. **Effect of adenovirus-mediated supplement of adiponectin on the injury-induced neointimal thickening.** *A*, representative immunoblot analysis of plasma adiponectin in WT or APN-KO male mice (8–11 weeks old) on the 4th day after injection with either Ad-βgal or Ad-APN. *B*, representative hematoxylin-eosin-stained sections of femoral arteries from Ad-βgal-treated and Ad-APN-treated APN-KO mice at 2 weeks after injury. *Arrows* indicate intims. *C*, quentitative analysis of the I/M ratio of injured femoral arteries from Ad-βgal-treated WT (n = 5, ± S.E.), Ad-APN-treated WT (n = 5, ± S.E.), Ad-βgal-treated APN-KO (n = 5, ± S.E.), and Ad-APN-treated APN-KO mice (n = 5, ± S.E.). The I/M ratio was significantly greater in Ad-βgal-treated APN-KO mice than in Ad-βgal-treated WT mice (#, p = 0.002, t test) and decreased by Ad-APN treatmant in APN-KO mice (*, p = 0.001, t test).

proliferation of vascular smooth muscle cells (VSMCs) by immunohistochemical detection of BrdUrd-labeled VSMCs in the sections from each femoral srtery at 2 weeks after injury (Fig. 2*A*). Qnantitative data of proliferation index revealed that intimal VSMC proliferation induced by vescular injury wss ~2-fold greater in KO mice than in WT mice (p = 0.001) (Fig. 2*B*). In non-injured arteries of both WT and KO mice, BrdUrd-lebeled VSMCs were barely detectable (data not shown). These deta demonstrate that deficiency of adiponectin in KO mice caused severe neointimal hyperplasia after artery injury, suggesting an inhibitory effect of sdiponectin on the proliferation of VSMCs in injured arteries.

Adenovirus-mediated Supplement of Adiponectin Attenuates Neointimal Thickening in Injured Arteries—To investigete the *in vivo* effects of adiponectin supplement on neointimal hyper-

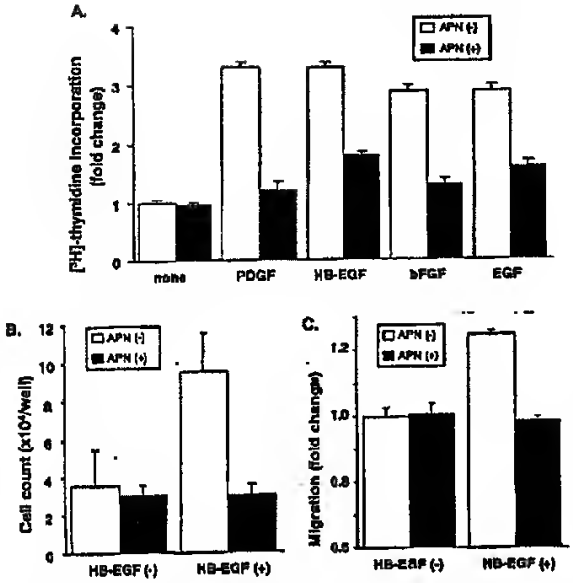


FIG. 4. **Inhibitory effect of adiponectin on the growth factor-induced proliferation and migration of cultured smooth muscle cells.** *A*, effect of adiponectin on DNA synthesis induced by PDGF, HB-EGF, basic FGF, and EGF in cultured HASMCs. HASMCs were treated with (*solid bars*) or without adiponectin (30 μg/ml) (*open bars*) either in the absence or presence of the indicated growth factors (10 ng/ml each) for 24 h, and DNA synthesis was calculated by measuring the incorporation of [³H]thymidine. *B*, effect of adiponectin on cell number of HASMCs stimulated by HB-EGF. HASMCs were treated without or with 10 ng/ml HB-EGF in the absence (*open bars*) or presence (*solid bars*) of 30 μg/ml adiponectin for 5 days. *C*, effect of adiponectin on migration of HASMCs stimulated by HB-EGF. HASMCs were incubated in the Boyden chamber at 37 °C for 4 h without or with HB-EGF (10 ng/ml) in the absence (*open bars*) or presence (*solid bars*) of adiponectin (30 μg/ml). The data (mean ± S.E.) from three independent experiments are shown.

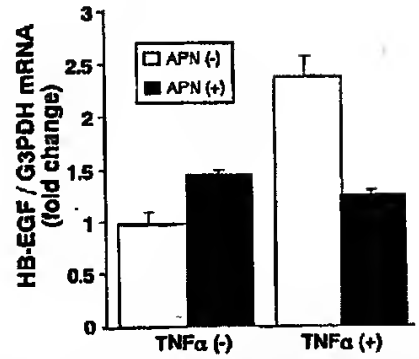


FIG. 5. **Inhibitory effect of adiponectin on the TNFα-induced expression of HB-EGF mRNA in cultured endothelial cells.** HAECs were pretreated without (*open bars*) or with (*solid bars*) adiponectin (30 μg/ml) for 18 h and then stimulated by adding TNFα (10 ng/ml) or vehicle for 2 h. HB-EGF and glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*) mRNA expression was measured by real-time quantitative reverse transcriptase-PCR using SYBR Green I as a double-stranded DNA-specific dye. The data (mean ± S.E.) from three independent experiments are shown.

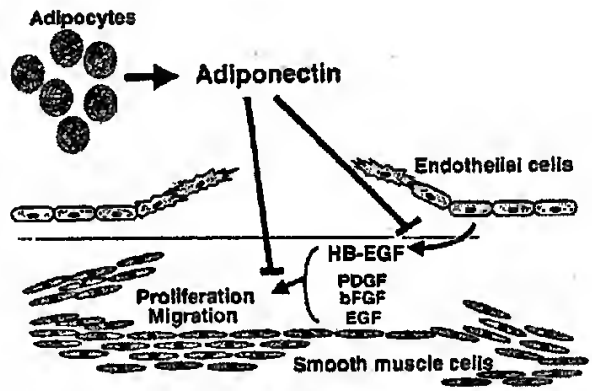


FIG. 6. **Model illustrating how adiponectin exerts a preventive effect on vascular stenosis in the injured artery.** *bFGF*, basic FGF.

plasia induced by vascular injury, we constructed recombinant adenovirus producing mouse adiponectin. WT and KO mice were infected with Ad-βgal or Ad-APN prior to vascular injury. Ad-APN infection resulted in a 2–3-fold increase in plasma levels of adiponectin on the 4th day after adenoviral injection in both WT and KO mice compared with those in Ad-βgal-infected WT mice (Fig. 3*A*). Hematoxylin-eosin-stained sections of the injured femoral arteries of adenovirus-treated KO mice showed that injection of Ad-APN resulted in the suppression of neointimal formation induced by vascular injury (Fig. 3*B*). Quantitative analysis of these sections revealed that the I/M ratio of femoral arteries of Ad-βgal-treated KO mice was significantly greater than that of Ad-βgal-treated WT mice ($p = 0.002$) (Fig. 3*C*) as was shown in mice without adenoviral infection in Fig. 1. In the KO mice, the adenovirus-mediated production of adiponectin in plasma attenuated the increase of I/M ratio to the levels of WT mice ($p = 0.001$) (Fig. 3*C*). These results demonstrate that adiponectin supplement could reverse neointimal hyperplasia in KO mice.

Adiponectin Suppresses Growth Factor-induced Proliferation and Migration of Cultured VSMCs—*In vitro* experiments provided strong evidence that adiponectin exerts suppressive effects on VSMC proliferation. PDGF, HB-EGF, basic FGF, and EGF have potent mitogenic activities on HASMCs. Adiponectin treatment attenuated growth factor-induced DNA synthesis in HASMCs (Fig. 4*A*). The inhibitory effect of adiponectin on HASMC proliferation induced by HB-EGF was directly shown by counting the cell number (Fig. 4*B*). In addition, adiponectin also suppressed HB-EGF-induced migration of HASMCs (Fig. 4*C*).

Adiponectin Attenuates the Expression of HB-EGF mRNA in Cultured Endothelial Cells—Next we investigated whether adiponectin could suppress the production of HB-EGF in endothelial cells. Adiponectin treatment completely blocked the TNFα-mediated increase of HB-EGF mRNA in HAECs (Fig. 5).

DISCUSSION

In the current study, we demonstrated that adiponectin-null mice exhibited augmented intimal proliferation in mechanically injured vascular walls. Adenovirus-mediated supplement of adiponectin improved the intimal thickening in KO mice to the WT level. How does adiponectin suppress intimal thickening? Fig. 6 illustrates a working model based on the results of our *in vivo* and *in vitro* experiments described in the present study. Adiponectin suppressed the expression of HB-EGF in stimulated endothelial cells of injured vascular wall and also the proliferation and migration of smooth muscle cells stimulated by various growth factors such as PDGF, basic FGF, EGF, and HB-EGF. These suppressive effects of adiponectin on the production and action of growth factors in vascular wall should explain the mechanism for the suppressive action of adiponectin on the vascular stenosis and indicate that it could prevent

injury-induced intimal thickening.

Plasminogen activator inhibitor-1 and HB-EGF are vasoactiva substances produced by adipose tissue, elthough these subatances are not adipose-spacific. Both factora are considered to promote the development of vascular diseases in obesity (6, 7). Contrary to these factors, the plasma concentration of adipose-specific adiponectin is lower in obese subjects and patients with coronary ertery disease (11, 12). Tha present study demonstrated *in vivo* and *in vitro* that adiponectin suppressed VSMC proliferation. Taken togather, adipose tissue secretes both the offense molecules (plasminogen activator inhibitor-1 and HB-EGF) and the defense molecule (adiponectin) into the blood stream, reaching the vascular well. Then, in obesity, both the increase of offense molecules and decrease of defense molecule(s) in plasma should aggravate vascular diseases. Considering tha adipose specificity, adiponectin should play a major role in tha adipo-vescular axis.

Recant studies have identified the role of various molecules darived from adipose tissue in tha development of insulin resistance. These includa TNFα, leptin, and resistin (3–5). More recently adiponectin treatment has been shown to improve fatty ecid oxidation and insulin resistance in diabetic animals (23, 24). Adiponectin-null mice show normal insulin sensitivity under a regular diet but severe insulin resistance under a high fat/high sucrosa diet (17). Interestingly, subjects carrying a missensa mutation in the adiponectin gene associated with hypoadiponectinemia exhibit the phanotypa of the metabolic syndroma, including insulin rasistance and coronary artery disaasa (25). Thasa findings suggest that hypoadiponectinamia associated with obesity is located upstream of metabolic syndroma in the pathophysiology. In tha present study, adiponectin-null mice showed profound neointimal hyperplasia despite normal glucosa and lipid metabolism. Our resnlts indicate that injury-induced neointimal formation does not accelerate aa a result of abnormalities of glucosa and lipid metabolism but is directly caused by adiponectin deficiency. Therapeutic approaches that increasa plasma adiponectin concentration could be useful in preventing restenosis after vascular intervention.

Acknowledgments—Ws thank H. Kato, F. Katsube, and S. Tanaka for excellent technical assistance.

REFERENCES

1. Spiegalman, B. M., and Flier, J. S. (2001) *Cell* 104, 531–543
2. Friedman, J. M. (2000) *Nature* 404, 632–634
3. Friedman, J. M., and Halaas, J. L. (1998) *Nature* 395, 763–770
4. Hotamisligil, O. S., Shargill, N. S., and Spiegalmaa, B. M. (1993) *Science* 259, 87–91
6. Steppan, C. M., Bailey, S. T., Bhat, S., Brown, E. J., Banerjee, R. R., Wright, C. M., Patel, H. R., Ahima, R. S., and Lazar, M. A. (2001) *Nature* 409, 307–312
6. Shimomura, I., Funahashi, T., Takahashi, M., Maeda, K., Kotani, K., Nakamura, T., Yamashita, S., Miura, M., Fukuda, Y., Takamura, K., Tokunaga, K., and Matsuzawa, Y. (1996) *Nat. Med.* 2, 800–803
7. Matsumoto, S., Kishida, K., Shimomura, I., Maeda, N., Nagaretani, H., Matsuda, M., Nishizawa, H., Kihara, S., Funahashi, T., and Matsuzawa, Y. (2002) *Biochem. Biophys. Res. Commun.* 292, 781–786
8. Maeda, K., Okubo, K., Shimomura, I., Funahashi, T., Matsuzawa, Y., and Matsubara, K. (1996) *Biochem. Biophys. Res. Commun.* 221, 286–289
9. Scherer, P. E., Williams, S., Fogliano, M., Baldini, G., and Lodish, H. F. (1995) *J. Biol. Chem.* 270, 26746–26749
10. Hu, E., Liang, P., and Spiegelman, B. M. (1996) *J. Biol. Chem.* 271, 10697–10703
11. Arita, Y., Kihara, S., Ouchi, N., Takahashi, M., Maeda, K., Miyagawa, J., Hotta, K., Shimomura, I., Nakamura, T., Miyaoka, K., Kuriyama, H., Nishida, M., Yamashita, S., Okubo, K., Matsubara, K., Muraguchi, M., Ohmoto, Y., Funahashi, T., and Matsuzawa, Y. (1999) *Biochem. Biophys. Res. Commun.* 257, 79–83
12. Ouchi, N., Kihara, S., Arita, Y., Maeda, K., Kuriyama, H., Okamoto, Y., Hotta, K., Nishida, M., Takahashi, M., Nakamura, T., Yamashita, S., Funahashi, T., and Matsuzawa, Y. (1999) *Circulation* 100, 2473–2476
13. Zoccali, C., Mallamaci, F., Tripepi, G., Benedetto, F. A., Cutrupi, S., Parlongo, S., Malatino, L. S., Bonanno, G., Seminara, G., Rapisarda, P., Fatuzzo, P., Buemi, M., Niccoia, G., Tanaka, S., Ouchi, N., Kihara, S., Funahashi, T., and Matsuzawa, Y. (2002) *J. Am. Soc. Nephrol.* 13, 134–141
14. Okamoto, Y., Arita, Y., Nishida, M., Muraguchi, M., Ouchi, N., Takahashi, M., Igura, T., Inui, Y., Kihara, S., Nakamura, T., Yamashita, S., Miyagawa, J., Funahashi, T., and Matsuzawa, Y. (2000) *Horm. Metab. Res.* 32, 47–50
15. Ouchi, N., Kihara, S., Arita, Y., Okamoto, Y., Maeda, K., Kuriyama, H., Hotta, K., Nishida, M., Takahashi, M., Muraguchi, M., Ohmoto, Y., Nakamura, T., Yamashita, S., Funahashi, T., and Matsuzawa, Y. (2000) *Circulation* 102, 1296–1301
16. Ouchi, N., Kihara, S., Arita, Y., Nishida, M., Matsuyama, A., Okamoto, Y., Ishigami, M., Kuriyama, H., Kishida, K., Nishizawa, H., Hotta, K., Muraguchi, M., Ohmoto, Y., Yamashita, S., Funahashi, T., and Matsuzawa, Y. (2001) *Circulation* 103, 1057–1063
17. Maeda, N., Shimomura, I., Kishida, K., Nishizawa, H., Matsuda, M., Nagaretani, H., Furuyama, N., Kondo, H., Takahashi, M., Arita, Y., Komuro, R., Ouchi, N., Kihara, S., Tochino, Y., Okutomi, K., Horie, M., Takeda, S., Aoyama, T., Funahashi, T., and Matsuzawa, Y., (2002) *Nat. Med.* 8, 731–737
18. Sata, M., Maejima, Y., Adachi, F., Fukino, K., Saiura, A., Sugiura, S., Aoyagi, T., Imai, Y., Kurihara, H., Kimura, K., Omata, M., Makuuchi, M., Hirata, Y., and Nagai, R. (2000) *J. Mol. Cell. Cardiol.* 32, 2097–2104
19. Sata, M., Saiura, A., Kunisato, A., Tojo, A., Okada, S., Tokuhisa, T., Hirai, H., Makuuchi, M., Hirata, Y., and Nagai, R. (2002) *Nat. Med.* 8, 403–409
20. Karas, R. H., Schulten, H., Pare, G., Aronovitz, M. J., Ohlsson, C., Gustafsson, J. A., and Mendelsohn, M. E. (2001) *Circ. Res.* 89, 534–539
21. Hishikawa, K., Oemar, B. S., Tanner, F. C., Nakaki, T., Fujii, T., and Luscher T. F. (1999) *Circulation* 100, 2108–2112
22. Yu, S.-M. Tsai, S.-Y., Guh, J.-H., Ko, F.-N., Teng, C.-M., and Ou, J. T. (1996) *Circulation* 94, 547–554
23. Yamauchi, T., Kamon, J., Waki, H., Terauchi, Y., Kubota, N., Hara, K., Mori, Y., Ide, T., Murakami, K., Tsuboyama-Kasaoka, N., Ezaki, O., Akanuma, Y., Gavrilova, O., Vinson, C., Reitman, M. L., Kagechika, H., Shudo, K., Yoda, M., Nakano, Y., Tobe, K., Nagai, R., Kimura, S., Tomita, M., Froguel, P., and Kadowaki, T. (2001) *Nat. Med.* 7, 941–946
24. Berg, A. H., Combs, T. P., Du, X., Brownlee, M., and Scherer, P. E. (2001) *Nat. Med.* 7, 947–953
25. Kondo, H., Shimomura, I., Matsukawa, Y., Kumada, M., Takahashi, M., Matsuda, M., Ouchi, N., Kihara, S., Kawamoto, T., Sumitsuji, S., Funahashi, T., and Matsuzawa, Y. (2002) *Diabetes* 51, 2326–2326